STUDY OF PREVALENCE, RISK FACTORS, AND LIFETIME IMPACTS OF INFECTION WITH BOVINE LEUKEMIA VIRUS IN THE CANADIAN DAIRY INDUSTRY

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Department of Health Management
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ABSTRACT

The overall goal of the research described in this thesis was to lay a proper foundation for designing and conducting efficient control and eradication programs for infection with bovine leukemia virus (BLV) in the Canadian dairy industry.

The objective in Chapter 2 was to identify potentially important risk factors for BLV infection in Canadian dairy herds. Of 272 study herds, from 8 provinces of Canada and tested during 1998-2003, 78% were BLV-positive. Over 15 management determinants for the infection were evaluated. Herds with clinical cases of leukosis during the 12 months prior to sampling, as well as herds which purchased animals with unknown BLV infection status in the last five years, had a significantly increased proportion of BLV-positive cows. Herds from eastern provinces and those not purchasing cows in the last five years were more likely to be free from BLV compared to western provinces and farms purchasing cows in the last five years.

The objective in Chapter 3 was to determine the lifetime effects of BLV infection on milk production and longevity of dairy cows in Canada. Overall, 4052 cows from 348 herds were enrolled in a historical cohort study, based on test results from 1998-2003 and lactation and culling records post-testing until 2013. Positive cows to BLV had consistently greater probability of being culled (or dying) than the negative cows (over lifetime lactations 2-7). Only BLV-positive cows with short longevity (2 and 3 lifetime lactations) had a significantly lower lifetime milk production compared with their negative counterparts. As the cows lived longer (> 3 lactations), the difference in milk production between the two cohorts was no longer significant.

The objectives of Chapter 4 were: 1) to assess the potential for carryover of antibodies against BLV in milk samples obtained from shared meters; and 2) to determine if adjustment of the diagnostic test cut-off value would improve the test characteristics for meter-collected milk ELISA results. The study included 236 paired milk samples from 8 dairy farms in Prince Edward Island collected in 2013. Two simultaneous milk samples, one hand-collected at the beginning of milking, and the other from the corresponding milk meter, were taken from all lactating cows that were milked at the selected meters. The sequence of cows using each meter was recorded. Carryover of BLV antibodies at shared milk meters was significant. For low-titer cows, the carryover effect was positively associated with the titer of the preceding cows. This could result in generating false-positive results in the BLV antibody-ELISA test on meter-collected samples from dairy herd improvement (DHI) procedures. Based on a new optimal cut-point, a suspicious category on the ELISA titers was defined, and a retest on the samples falling within this range was recommended to reduce the false positive rate.

The objectives for Chapter 5 were: 1) to determine the prevalence of BLV infection at the herd level using a bulk-tank milk (BTM) antibody ELISA in the Maritime region of Canada ; and 2) to develop applied statistical models for predicting within-herd prevalence of BLV infection using the BTM antibody levels. To detect BLV infection and the antibody levels, a census was implemented on BTM samples from all dairy farms in the Maritime region in 2013 (3 monthly rounds of sampling on 623 farms). Another round of BTM sampling was coincided with individual cow sampling (all cows that contributed milk to the fourth BTM) in 90 selected herds. Herd-level prevalence of BLV in the Maritime region was 90.8%. In the individual testing, 30.4% of cows were

positive. The statistical models developed in this study were able to predict true within-herd prevalence of BLV reasonably well based only on the BTM results. The model including all BTM tests (4 rounds of sampling) as the predictor had the best fit, although the models using 2 and 3 BTM tests provided similar results to 4 repeated tests.

The focus for Chapter 6 was to assess the diagnostic performance of a commercially available ELISA for detecting BLV antibodies in BTM samples collected from dairy herds in Eastern Canada in 2013. Of 133 tested herds, 108 herds were found to be truly infected. At the resulting optimal cut-point, sensitivity and specificity of the BTM ELISA were estimated at 0.972 (0.921 – 0.994) and 1 (0.863 – 1), respectively.

With the high prevalence of BLV infection across Canada and its detrimental economic impacts, pursuing broad-based control programs is necessary. All of the findings in the present research could contribute to designing and conducting efficient BLV control programs.

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LIST OF ABBREVIATIONS

Abbreviation	
AB	Alberta
AGID	Agar gel immunodiffusion
AVC	Atlantic Veterinary College
BC	British Colombia
BLV	Bovine Leukemia Virus
BTM	Bulk Tank Milk
BVD	Bovine Viral Diarrhea
CI	Confidence Interval
DHI	Dairy Herd Improvement
DIM	Days in Milk
EBL	Enzootic Bovine Leukosis
ELISA	Enzyme Linked Immunosorbent Assay
HTLV-I	Human T-Lymphotropic Virus, Type I
kg	Kilogram
MB	Manitoba
MHC	Major Histocompatibility Complex
ml	Milliliter
MQM	Maritime Quality Milk
NB	New Brunswick
NL	Newfoundland and Labrador
NS	Nova Scotia
OD	Optical Density
ON	Ontario
PCR	Polymerase Chain Reaction
PE	Prince Edward Island
PL	Persistent Lymphocytosis
PP	Percent Positivity
QC	Quebec
RIA	Radioimmunoassay
ROC	Receiver Operating Characteristic (Curve)
S/P	Sample-to-positive ratio
SBL	Sporadic Bovine Leukosis
SCC	Somatic Cell Count
SD	Standard Deviation
Se	Sensitivity
SE	Standard Error
SK	Saskatchewan
Sp	Specificity
UPEI	University of Prince Edward Island
ZINB	Zero-Inflated Negative Binomial

CHAPTER 1

GENERAL INTRODUCTION

1.1 Enzootic bovine leukosis in dairy cattle

1.1.1 Background

Enzootic bovine leukosis (EBL) is known by different names, such as enzootic bovine lymphoma, bovine leukosis, infection with bovine leukemia virus (BLV), bovine lymphosarcoma, and bovine lymphoma. Leukosis in cattle was originally described in Germany in 1871 and reports of the disease in cattle became common following World War II and most countries raising cattle have reported the occurrence of the disease (Radostits et al., 2006).

The causative agent of the disease (BLV) is an oncogenic virus from the Retroviridae family, the Deltaretrovirus genus. The natural host for the virus is cattle. The virus causes a chronic B-cell proliferative disease in cattle and is an important model for human T-cell leukemia virus type 1 (HTLV-I) infection because of many shared molecular and biological features. Different studies have failed to find pathogenic effects of the virus in humans (Fenner et al., 2011). However, a recent case-control study suggested that the presence of BLV-related DNA in breast tissue specimens was associated with breast cancer (Buehring et al., 2015). Additional research is needed to examine this purported association further.

All breeds of cattle are susceptible to BLV infection (Radostits et al., 2006). Exposure of cattle to BLV results in four possible outcomes: 1) no infection; 2) establishment of a permanent infection and development of detectable antibodies for life (latent carriers; the most common form); 3) establishment of a permanent infection and development of persistent lymphocytosis (PL); or 4) establishment of a permanent infection and development of malignant lymphoma (cancer with or without PL). Whether the animal becomes infected or develops any other forms of the disease mostly depends on the host's genetic constitution, immune status, and the infective dose of the virus (Da et al., 1993; Kabeya et al., 2001). Approximately, 30% of the infected cows proceed to the PL stage and fewer than 5% will eventually develop malignant lymphoma during the typical lifespans of dairy cattle (Schwartz and Lévy, 1994).

1.1.2 Prevalence

Many European countries, including the UK, France, Germany, Spain, the Scandinavian countries, Belgium, Switzerland, and The Netherlands, are officially free from EBL (Annual EU report, 2013). Some other countries, such as Japan, the United States, and Argentina, have actively been working on addressing their BLV problems in order to develop cost-effective programs for their dairy industries (Ott et al., 2003; Monti et al., 2007; Murakami, 2009; Rodr et al., 2011).

In Canada, a number of serological surveys have been conducted in different provinces to estimate the prevalence of BLV infection. In 1980, the national prevalence of BLV infection in Canadian dairy herds was estimated at 40.5%, while only 9.3% of tested cattle were positive (Samagh and Kellar, 1982). However, 15 to 20 years later, infection levels appeared to have substantially increased. Sargeant et al. (1997) stated that

69.6% of the 102 tested dairy herds, and 23% of the 1330 tested cows in Ontario were positive to BLV. VanLeeuwen et al. (2001) reported that 70% of herds in the Maritime region of Canada had at least one infected cow, while the prevalence of infection at the cow level was estimated at 20.8%. Similar studies have revealed a high prevalence of BLV infection in Western Canada (VanLeeuwen et al., 2005; VanLeeuwen et al., 2006; Scott et al., 2006).

1.1.3 Transmission and risk factors

For a susceptible host to become infected with BLV, virus-infected B lymphocytes must gain access to the vascular and/or lymphatic systems. The usual method for spread of BLV infection in cattle populations is horizontal transmission, through direct and indirect exposure of susceptible animals to the infected lymphocytes from blood or, less likely, milk (Ferrer, 1979; Radostits et al., 2006). Some of the typical iatrogenic routes of transmission are through contaminated surgical instruments, dehorning gouges, ear tattooing pliers, rectal sleeves, and syringes and hypodermic needles used between infected and susceptible animals without disinfection. Blood-sucking insects (e.g. stable fly) may also be involved in the transmission cycle of the virus through direct transmission of virus from one animal to another. Although transplacental transmission of BLV has been documented, it seems to be relatively infrequent (De Jong et al., 2007; Smith, 2009). Congenital infection occurs in 4-8% of calves born to BLV-seropositive cows in naturally infected herds (Radostits et al., 2006). The contributions of different modes of transmission depend on the frequency and nature of BLV exposure, along with the prevalence of infection within the herds (Gutiérrez et al., 2011).

Prevalence of the infection is positively associated with increasing age. Kale et al. (2007) reported that the proportion of infected cows in a Turkish dairy herd gradually increased from 52.6% in the first-lactation cows to 66.6% in the fifth-lactation cows. Erskine et al. (2012c) proposed a BLV herd profile as a practical tool to determine age-stratified prevalence of BLV infection in dairy herds. They indicated that BLV within-herd prevalences (in 113 Michigan dairy herds) were 18.5, 28.8, 39.2, and 44.8% in 1st, 2nd, 3rd, and ≥4th lactation cows, respectively.

Poor biosecurity measures, such as introduction of infected cattle to a herd, have a significant effect on the subsequent prevalence of infection and the occurrence of clinical disease. Any environmental factor or management practice which exposes newborn calves to infected blood will increase the risk of infection in the calves; these exposures include prolonged close contact between the cow and calf immediately after parturition, or any kind of blood-contaminated management interventions such as using contaminated needles (Kobayashi et al., 2010; Erskine et al., 2012b).

Colostrum-based transmission of BLV could also pose a risk of infection to neonatal calves, although the significance and magnitude of this risk still remains uncertain (Kanno et al., 2014). It has been reported that colostrum and pooled milk (from infected dams) increase the risk of BLV transmission, whereas the antibodies in colostrum decrease the risk of infection (Romero et al., 1983). Nagy et al. (2007) claimed that calves born to BLV-positive cows would be exposed to BLV during parturition, but the administration of colostrum from the infected dam would exert a substantial protective effect due to the passive antibodies (Nagy et al., 2007). In another study, feeding dams' colostrum to their calves was associated with a decrease in the within-herd

seroprevalence of BLV (Kobayashi et al., 2010). Kanno et al. (2014) suggested that freezing treatment for colostrum provides a viable means of inactivating the infectivity of BLV-infected lymphocytes; however, additional studies to identify the optimal conditions for the treatment of pooled colostrum are still required.

The dynamic of the infection in a cow and the spread of virus within a herd are highly dependent on genetic factors of both the virus and the host. Progression of infection in a cow to PL is controlled by the bovine major histocompatibility complex (MHC). Resistance factors in some animals control the rate of progression of the infection and result in reduced numbers of viral-infected cells and lower cell counts, which would tend to decrease the risk of BLV transmission (Jubb et al., 1993; Bartlett et al., 2014).

1.1.4 Economics

Infection with BLV imposes substantial economic loss to dairy herds and countries with high prevalence of the infection (e.g. Canada and the USA). Major adverse economic impacts of BLV infection include: premature culling, death, and condemnation of carcasses at slaughter due to lymphoma, production loss, lower reproductive efficiency, impaired immune function and susceptibility to other pathogens, as well as trade restrictions imposed on infected cattle and their products (Sandev et al., 2000; Bartlett et al., 2014). However, the effects of subclinical BLV infection on production, reproductive performance, and longevity/culling rate have been debated in the literature.

A number of studies have failed to demonstrate a statistically significant association between BLV infection and milk production in dairy cows and herds (Landston et al., 1978; Huber et al., 1981; Brenner et al., 1989; Da et al., 1993; Tiwari et

al., 2007; Sorge et al., 2011). In contrast, detrimental effects of BLV infection on production have been reported by others (Emanuelson et al., 1992; Sargeant et al., 1997; D'Angelino et al., 1998; Ott et al., 2003; Erskine et al., 2012a). For instance, Erskine et al. (2012) reported that each 0.1 increase in the proportion of positive cattle in 104 Michigan dairy herds was significantly associated with a 115 kg decrease in 12-month rolling herd average milk yield. Ott et al. (2003), in a large US study, showed that herds with seropositive cows produced 218 kg/cow less milk compared to negative herds. Da et al. (1993) demonstrated that positive herds produced 3% less milk per cow. VanLeewen et al. (2010) suggested a 7% lower conception rate and longer calving interval in seropositive cows compared with their negative counterparts, whereas some other researchers could not show any significant association between BLV infection and reproduction measures (Brenner et al., 1989; Emanuelson et al., 1992). Although some researchers have reported a negative association between BLV infection status and longevity of dairy cows (Pollari et al., 1992; Emanuelson et al., 1992; Tiwari et al., 2005; Bartlett et al., 2013), they were not able to establish a strong association.

Measured at the herd level, the direct production losses from EBL in the Canadian Maritimes (including provinces of Prince Edward Island (PE), New Brunswick (NB), and Nova Scotia (NS)) were conservatively estimated at $806 per year in an average 50-cow herd. This does not include costs associated with lost sales of genetically superior purebred cattle, which are likely more substantial than the direct production impacts (Chi et al., 2002). Furthermore, economic loss per case of lymphoma was estimated to be $412 in another study (Rhodes et al., 2003). Annual economic losses to the US dairy industry associated with BLV were estimated at $285 million for producers and $240 million for

consumers; additionally, the subclinical impact of BLV infection on cow longevity was not included in those estimations (Ott et al., 2003; Bartlett et al., 2014).

Most of bull studs protect their export markets by only buying BLV-negative bulls because many countries only import semen from studs that are entirely free from BLV (Losinger, 2006; Radostits et al., 2006). In Canada, BLV-seropositive bulls are barred entry into artificial insemination units. As the concern for BLV increases within the world market, it is more likely that cattle buyers confine their purchases only to BLV-negative replacements.

A herd with a high prevalence of BLV infection may experience significant losses resulting from a high number of clinical cases that have no salvage value. Occurrence of the disease can be a major cause of economic loss in high producing elite dairy herds, where pedigreed livestock are sold. In these pedigreed herds, individual animals are kept to a much older age than in average commercial herds, and because of the increased prevalence of lymphoma in cows over 5 years of age, the death losses are likely to be severe in the exact group of cows, which is critical to the success of a herd. In addition, there is a considerable negative effect on the salability of stock from a herd known to have a disease in which genetic susceptibility is thought to play an important role (e.g. BLV) (Radostits et al., 2006; Smith, 2009).

1.1.5 Diagnosis

1.1.5.1 Lymphoma

Clinical disease is characterized by the occurrence of multicentric lymphoma, with tumors developing rapidly in many sites with an accompanying great variation in clinical signs and syndromes. This form is rarely seen in animals less than 2 years of age and is most common in the 4-8 year age group. Some of the predominant clinical signs

include enlargement of superficial lymph nodes, digestive tract lesions, as well as cardiac, nervous, and urogenital systems involvement. The clinical signs and the duration of the illness vary with the number and importance of the sites involved and the speed with which the tumor masses grow. Neoplastic tumors are identified by histological examination of a biopsy specimen, or at necropsy (Schwartz and Lévy, 1994; Smith, 2009).

1.1.5.2 Persistent lymphocytosis

Persistent lymphocytosis (a stable increase in the number of circulatory lymphocytes) without clinical signs usually occurs earlier in life, but rarely before 2 years of age. Many cows remain in the preclinical stage for years, often for their complete productive lifetime without any apparent reduction in performance, but clinical disease eventually appears in a proportion of these cows. Persistent lymphocytosis is identified through hematologic examinations by observing a permanent increase in absolute number of peripheral blood circulating B-lymphocytes (above 10,000/mm^3) (Radostits et al., 2006; Smith, 2009).

1.1.5.3 Common laboratory techniques for detecting BLV infection

Diagnosis of the infection is made by standard serological (e.g. AGID, RIA, and ELISA) or virus detection techniques (e.g. PCR). Cattle infected with BLV will produce antibodies against the major internal (p24) and envelope (gp51) virion proteins in their serum and milk; hence, antibody-based tests are commonly used for the diagnosis of BLV infection in cattle over 6 months of age (Radostits et al., 2006). Once cattle become infected with BLV, they remain infected for life and generate a continuous antibody

response. This characteristic adds to the validity of antibody-based diagnostic techniques for BLV (Monti et al., 2007; Kobayashi et al., 2010).

From a cost perspective, milk ELISA is a desirable method for BLV-antibody detection in large-scale herd surveillance programs which has often been used for detection of infected cows and herds (Erskine et al., 2012c). Current commercial ELISA kits offer excellent accuracy (nearly 100%) at the individual level application. Both serum and milk samples could be used for detection of antibodies. Moreover, the excellent analytical sensitivity of ELISA for pooled serum/milk samples allows the detection of infected herds with low prevalence (Mammerickx et al., 1985; Ridge and Galvin, 2005; Radostits et al., 2006).

Using bulk-tank milk (BTM) samples, collected by the dairy herd improvement (DHI) companies, has become one of the most convenient and economically efficient mechanisms for screening for important infectious diseases in dairy cattle (Houe et al., 1995; Attalla et al., 2010; Sorge et al., 2011). For instance, BTM ELISA has frequently been applied to the surveillance of EBL, Johne's disease, and bovine viral diarrhea (Niskanen, 1993; Bitsch and Ronsholt, 1995; Reber et al., 2012; Nielsen and Toft, 2014).

1.1.5.4 Sporadic bovine leukosis

A clear distinction must be made between EBL and sporadic bovine leukosis (SBL). No association has been demonstrated between SBL and the presence of an infectious agent (including BLV). Sporadic bovine leukosis mainly affects animals under 3 years of age and manifests itself in one of the three following forms: 1) juvenile form in calves, less than 6 months, characterized by multiple lymph node enlargement; 2) thymic form in yearlings, less than 2 years, characterized by a swelling in the neck causing bloat

and edema; and 3) cutaneous form, in cattle 1–3 years old, characterized by the development of nodes and plaques in the skin (Radostits et al., 2006; Smith, 2009).

1.1.6 Immunological implications

The most obvious immunologic effect of BLV infection is a peripheral blood lymphocytosis, which may be indicative of the start of altered immune function. The virus is lymphotropic and is believed to cause peripheral blood mononuclear cell proliferation and altered apoptosis and cytokine production. Infection with BLV induces accumulation of B-lymphocytes in blood and lymphoid tissue with concurrent decreases in the percentages of T-lymphocytes (Swenson et al., 2013). Infected T cells increase the expression of immunoinhibitory receptors, which in turn enhance the ability of pathogens that cause chronic infections to evade immune defenses. This expression of immunoinhibitory receptors is positively associated with proviral load (Bartlett et al., 2014).

Lymphocytosis is the standard predictor of disease progression, although it is not necessarily associated with pathogenic change (Rodriguez et al., 2011). However, several reports have suggested that the use of proviral load as an alternative indicator for disease progression (Rodriguez et al., 2009; Jimba et al., 2010). When lymphocyte count is not available, the lactation number of infected cattle may provide a crude proxy in that older animals are more likely to have infections of longer duration and, therefore, their BLV infections are more likely to have progressed to the PL stage. Independent of cow age, BLV ELISA titers may also indicate a progression of BLV pathogenesis toward immunosuppression (Bartlett et al., 2013). Therefore, cows with higher viral load and/or

circulatory antibody levels could have a greater potential for spreading the infection within a herd.

1.1.7 Management, control, and eradication

Bartlett et al. (2014) described all of the reasonable options for dealing with BLV infection in the US dairy herds which could fundamentally be extended to the Canadian dairy herds. The disease can be eradicated from a herd and even a country, or controlled at a low level of prevalence. The option chosen depends primarily on the prevalence of infection within a herd, the value of the animals in the herd, and whether a governmental indemnity is offered for seropositive cows which are culled and sent to slaughter (Bartlett et al., 2014).

For a comprehensive control/eradication program for BLV infection, four main options or a combination of those are suggested: 1) doing nothing; 2) taking appropriate control measures; 3) testing and segregation of positive cows; and 4) testing and removal (to eradicate BLV). In Canada and the US, it is considered cost-prohibitive to test and slaughter all seropositive cattle in many herds because of the high prevalence in those herds. Furthermore, many seropositive cows are valuable pedigreed animals, and there are no indemnity programs in place. Thus, all control and eradication programs in these countries are herd-based and strictly voluntary. Livestock producers are mostly willing to adopt control measures because of the economic losses associated with export restrictions if their cattle are infected, and the losses due to the occasional clustering of cases of lymphoma (Rodríguez et al., 2011).

Determining the approximate number of infected cows in a herd can be of particular importance when a decision on adopting the most suitable control or

eradication strategy is to be made. For instance, if only a few positive cows in a herd were present, eradication measures (test and removal) could be economically justified. If a low prevalence (e.g. 10%) of the infection was expected, then a test and segregation strategy could be desirable. The decision rules (farm protocols) for farms with estimated prevalence above 20-30% would be similar; usually including standard management practices in order to reduce the within-herd transmission of BLV (Casal et al., 1990). For herds with medium-to-high levels of within-herd prevalence (e.g. > 20-30%) management strategies without removal or segregation for controlling BLV are preferred due to the considerable cost (and/or impracticality) of removal and segregation schemes (Sandev et al., 2000; Bartlett et al., 2014). Commonly cited management practices for minimizing the transmission of BLV in dairy herds are as follows:

1. Using single-use or disinfected needles and syringes between cows;
2. Using sterile or disinfected equipment for medical/management interventions (e.g. surgery, tattooing, dehorning);
3. Using single-use or disinfected rectal palpation sleeves;
4. Avoiding contacts between newborn calves and positive animals;
5. Not feeding calves with milk or colostrum from positive cows;
6. Minimizing contacts between different age-groups of animals;
7. Using artificial insemination (AI) for breeding;
8. Reducing the populations of blood-sucking insects on the farm;
9. Segregating or culling BLV-positive cows;
10. Purchasing BLV-negative replacements.

Vaccination could be an effective strategy to control BLV infection (especially in high-prevalence regions); however, no commercial vaccine for BLV is currently available (Gutiérrez et al., 2014). Many previous attempts to develop an effective vaccine against BLV have been unsuccessful, mainly due to incomplete or transient stimulation of the host immune response. Some promising results have recently been achieved in developing an attenuated, but replication competent, clone that protects against the virus in dairy herds (Gutiérrez et al., 2014). An ideal BLV vaccine would have to be non-infectious, non-oncogenic, and should not interfere with the tests commonly used to detect the infection (Radostits et al., 2006).

Another potential approach to reduce BLV infection in some valuable dairy herds could be through the selection of BLV-resistant cattle. Researchers have suggested different links between BLV infection and genetic components of cattle. Immune responsiveness and heritable resistance, or susceptibility to the infection, are believed to be influenced by the host MHC (Da et al., 1993; Radostits et al., 2006; Fenner et al., 2011).

In general, control or eradication of BLV infection in Canada should be given serious consideration due to:

- Very high prevalence of the infection at herd and cow levels and its rising trend over the past years (as discussed earlier);
- More countries becoming free from the infection or putting stringent control measures in place - this could lead to loss of many export markets and trade opportunities in the future;
- Growing knowledge of production impacts of BLV infection on cattle;

- Further concerns regarding the immune function of affected cattle which could result in increasing spread of opportunistic infections (e.g. mastitis) (Frie and Coussens, 2015);
- Increasing public concerns regarding the general health and welfare of cattle and the quality of their products (Bartlett et al., 2014); and
- Recent evidence of a possible link between BLV and breast cancer (Buehring et al., 2015).

1.2 Current deficiencies and challenges in controlling enzootic bovine leukosis

With respect to defining a comprehensive control program for EBL in Canada, some of the main challenges that the decision makers are facing include: inadequate awareness of the negative impacts and the current burden of the infection on the dairy industry, increasing trend of the prevalence in herds and cows, lack of a commercial vaccine, as well as costly surveillance methods. To address these challenges and justify the necessity for comprehensive control programs in Canadian dairy herds, a few fundamental steps must be taken: 1) determining current within-herd prevalence (for herd-specific plans) as well as herd-level prevalence (for regional or national programs) of the infection; 2) understanding substantial risk factors affecting the distribution of the infection within and between herds; 3) estimating the current economic impacts and consequences of the infection; and 4) defining the most pragmatic, time/cost-efficient control or eradication schemes. Being able to design and introduce efficient monitoring tools will assist in motivating authorities and producers to take decisive actions against BLV.

The prevalence of BLV infection has seen a pronounced rising trend over the past decades in Canada (as indicated in section 1.1.2); however, no united regional or national control/eradication programs have been pursued to deal with this increasingly important issue. In order to design effective control programs, identifying major risk factors of the infection is necessary. None of the previous studies has investigated a wide range of potential risk factors for BLV on a broad scale in Canada.

Production effects of BLV infection have been debated in the literature. Some of the reasons for obtaining inconsistent findings in the historic studies were: 1) the cross-sectional nature of those studies; 2) limited number of study cows or herds; 3) in some studies, disregarding the structure of data in the statistical analyses; and 4) applying production data restricted to the lactations in which BLV testing was performed or a few lactations later (as a result, not accounting for possible seroconversions and the progression of infection). In addition, there has not been any study investigating the lifetime impacts of the infection, which could be more relevant with regard to the chronic nature of BLV. To effectively address the BLV problem in every herd, region, or country, real-time costs of the infection must initially be estimated (Smith, 2009).

Another necessary step towards designing efficient control programs is to attain a real-time estimate of the number of infected cows in a herd because the management options are highly dependent on the within-herd prevalence (Bartlett et al., 2014). To do this, a census or at least a representative sample of cows from each dairy herd is required. This would not be appealing to the industry and farmers due to financial limitations and other important priorities. Hence, to encourage the producers towards adopting appropriate control measures, convenient and cost-efficient screening tools are needed.

The persistent nature of BLV infection, the continuous antibody response, and the absence of commercial vaccination make this infection a reasonable candidate for investigating quantitative prediction models for within-herd prevalence (Radostits et al., 2006; Gutiérrez et al., 2014). Therefore, quantitative examination of BTM samples could be an interesting, viable option for reducing costs and saving time/labour in future surveillance programs.

In order to increase the efficiency of monitoring schemes for important infectious diseases of dairy cattle, including BLV, milk samples collected by DHI companies are of interest. With increasing utilization of DHI diagnostic services on meter-collected samples, a legitimate concern has emerged regarding carryover or cross-contamination of the samples from shared milking equipment. With respect to sequential milk samples, carryover denotes the risk of inclusion of some residual milk from the previous cows in the subsequent milk samples (Ordolff, 1997; Lovendahl and Bjerring, 2006).Thus, an important question would be - to what extent carryover could influence the transfer of diagnostic targets (e.g. BLV-antibodies) in milk between cows, and would that amount of transferred material substantially obscure the accurate interpretation of the diagnostic tests on those samples? Diagnostic tests with high analytical sensitivity (e.g. ELISA and PCR) could produce considerable numbers of false positive results. Antibody-ELISA is the most commonly used test in current volunteer screening and surveillance programs for BLV and has an excellent analytical sensitivity (Florent et al., 1988; Joozani et al., 2012); hence, it is possible that even low quantities of carried antibodies could be detected by this method. The high rate of false results could in turn lead to making inappropriate management decisions, followed by prominent economic losses. There has

not been any study focused on the carryover of diagnostic targets in shared milk meters and its potential detrimental effects on the success of the current screening and surveillance programs.

In the current screening and volunteer control programs for BLV infection, commercial ELISA test kits have widely been used. Applying such tests, particularly to the pooled samples (e.g. BTM), might lead to variable levels of uncertainty in the results. Several factors, including study region, herd (pool) size, sampling procedures, and transfer process can potentially increase variability in test results. Therefore, it has been recommended that the validity of diagnostic tests should be evaluated in different populations before integrating the tests in large-scale control and eradication programs (Christensen and Gardner, 2000; Greiner and Gardner, 2000). However, the commercial BLV test which is currently being used in Eastern Canada has not been validated for its routine application as a diagnostic test.

1.3 Thesis objectives/Focus of research

The research described in this thesis was conducted to address the outlined deficiencies and concerns regarding BLV infection in Canada. The overall goal of this thesis was to lay a proper foundation for designing and conducting efficient control and eradication programs for BLV in Canada (by examining risk factors, productivity impacts, and efficient testing strategies for BLV). The specific objectives pursued in substantive chapters of this thesis are described below.

1.3.1 Herd-level risk factors for BLV infection

In Chapter 2, a cross-sectional study was conducted to identify some of the most important risk factors associated with seroprevalence of BLV in Canadian dairy herds.

This was the first time that herd-level risk factors for BLV was investigated in Canada on such a broad scale. The data used in this chapter were extracted from a comprehensive national survey of production limiting diseases, which took place between 1998 and 2003 in eight provinces of Canada.

1.3.2 Lifetime impacts of BLV on milk production and longevity

In Chapter 3, a historical cohort study was conducted to determine the lifetime effects of BLV infection on milk production and longevity of dairy cows in Canada. For this study, sub-groups within the national dataset were combined with longitudinal production data.

1.3.3 Carryover effects of BLV antibodies

In 2013, a new project was designed and conducted in the Maritime provinces to determine the present status of the infection, as well as to evaluate cost-efficient monitoring tools for BLV (Chapters 4-6). In Chapter 4, an observational study was designed: 1) to assess the potential for carryover of BLV antibodies in milk samples obtained from shared meters; and 2) to determine whether adjustment of the diagnostic test cut-off values would improve the test characteristics for meter-collected milk-ELISA results.

1.3.4 Predicting within-herd prevalence of BLV

In Chapter 5, a repeated survey was conducted: 1) to determine the prevalence of BLV infection at the herd level using a BTM antibody ELISA in the Maritime region of Canada ; and 2) to develop applied statistical models for predicting within-herd prevalence of BLV infection using the BTM antibody levels. The ultimate goal was to

reduce the hassles and costs that pertain to individual cow sampling in estimating the within-herd prevalence of the infection.

1.3.5 Validating an ELISA test at herd level application

In Chapter 6, combining data from the 2013 study with a similar project implemented in Quebec (in 2014), the diagnostic performance of a commercially available ELISA (Svanovir BLV gp51-Ab, Svanova, Uppsala, Sweden) for detecting BLV-antibodies in BTM samples was assessed. The primary goal was to validate the routine application of this test to BLV monitoring in Eastern Canada.

Finally, Chapter 7 is dedicated to summarizing the conclusions and discussing the research limitations and future directions for controlling BLV infection in Canada. In this chapter, a provisional example of a comprehensive BLV control program is presented.

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CHAPTER 2

HERD-LEVEL RISK FACTORS FOR INFECTION WITH BOVINE LEUKEMIA VIRUS IN CANADIAN DAIRY HERDS

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2.1 Abstract

Enzootic bovine leukosis (EBL) is an economically important infection of dairy cattle caused by bovine leukemia virus (BLV). The prevalence of infection in Canadian dairy herds is high and continues to increase; however, there has not been a national program to control BLV. This cross-sectional study was conducted to identify potentially important risk factors for BLV infection on Canadian dairy farms, which is a prerequisite to developing an effective control program.

During 1998 - 2003, based on a stratified two-stage random sampling process, 315 dairy farms from seven provinces of Canada were selected. Within each farm, 9 to 45 cows were bled and tested with a commercial serum ELISA kit for BLV-antibodies. A comprehensive questionnaire, targeting potentially important herd-level management indicators, was successfully administered in 272 herds. A zero-inflated negative binomial (ZINB) regression model was built to assess the potential associations between BLV seropositivity and a variety of herd-level factors.

Seventy-eight percent of the herds were identified as BLV-positive (had one or more test positive animals). In the negative-binomial part of the final ZINB model, herds with clinical cases of leukosis during the 12 months prior to sampling, as well as herds which purchased animals with unknown BLV infection status in the last five years, had a significantly larger proportion of BLV positive animals. Based on a significant interaction between two of the risk factors, changing gloves between cows during pregnancy examination was not statistically associated with lower proportion of infected cows compared with not changing gloves, in the western Canadian provinces. In the logistic part of the model, herds from eastern Canadian provinces and those not

purchasing cows in the last five years had increased odds of being free from BLV. The high prevalence of infection across Canada should be addressed through the development and implementation of a nationwide control program which will address the regional and herd-level risk factors for BLV infection identified in this study.

2.2 Introduction

Enzootic bovine leukosis (EBL) is an economically important infection of dairy cattle worldwide, which is caused by bovine leukemia virus (BLV). Clinical signs of the disease are not displayed by most infected cattle; fewer than 5% of them will eventually develop malignant lymphoma. Premature culling, death, and condemnation of carcasses at slaughter due to lymphoma, as well as trade restrictions imposed on infected cattle and their products are among the most significant losses attributed to the disease (Sandev et al., 2000; Bartlett et al., 2014).

The usual method for spread of BLV infection in cattle populations is horizontal transmission through direct and indirect (e.g. iatrogenic) exposure of susceptible animals to the infected lymphocytes from blood or less likely milk. Although transplacental transmission of BLV has been documented, it seems to be infrequent (Radostits et al., 2006; Gillet et al., 2013). The contribution of different haematogenous modes of transmission depends on the frequency and nature of BLV exposure, along with the prevalence of infection within the herds (Gutiérrez et al., 2011). In order to control the infection, it is necessary to properly determine and inhibit the important modes of transmission. Once cattle become infected with BLV, they usually remain infected for life and have a continuous antibody response (Monti et al., 2007; Kobayashi et al., 2010);

this characteristic of the infection adds to the validity of antibody-based diagnostic techniques.

The 2013 annual report of the European Union on bovine and swine diseases declared that many European countries including the UK, France, Germany, Spain, the Scandinavian countries, and the Netherlands were officially free from EBL (Annual EU report, 2013). Other countries, such as Japan, the United States, and Argentina, have been actively working on addressing their BLV problems in recent years in order to develop effective programs for their dairy industries. Control of EBL at the national level usually consists of one or more of the following three approaches: management interventions; test and segregation; and test and slaughter (Ott et al., 2003; Rodríguez et al., 2011; Murakami et al., 2011; Bartlett et al., 2014). Management interventions can only be effective if the most important management determinants are identified, well understood, account for a sizable attributable risk and allow easy and economical remediation (Erskine et al., 2012b).

In different provinces of Canada, there have been a number of serological studies which have estimated the prevalence and impact of BLV infection. In 1980, national prevalence of BLV infection in Canadian dairy herds was estimated at 40.5%, while only 9.3% of tested cattle were positive (Samagh and Kellar, 1982a). However, 15 to 20 years later, infection levels appeared to have increased, substantially. Sargeant et al (1997) indicated 69.6% of the 102 tested dairy herds, and 23% of the 1330 tested cows in Ontario were positive to BLV. VanLeeuwen et al (2001) reported that 70% of herds in the Maritime region of Canada (including provinces of Prince Edward Island, New Brunswick, and Nova Scotia – forming part of the database for the current study) had at

least one infected cow, while the prevalence of infection at the cow-level was estimated at 20.8%. Similar studies revealed a high herd-level prevalence of BLV infection across Canada (VanLeeuwen et al., 2005a; VanLeeuwen et al., 2006; Scott et al., 2006). Measured at the herd level, the direct production losses from EBL in the Maritime provinces have been conservatively estimated at $806 per year in an average 50-cow herd (Chi et al., 2002). This does not include costs associated with lost sales of genetically superior purebred cattle, which are likely more substantial than the direct production impacts.

Since there is no nationwide program for controlling EBL in Canada, this cross-sectional study was conducted to identify some of the most important risk factors associated with seroprevalence of BLV in Canadian dairy herds. This is the first time that herd-level risk factors for BLV have been investigated in Canada on such a broad scale.

2.3 Materials and methods

The data set was extracted from the Canada-wide surveys of production limiting diseases that took place between 1998 and 2003 on dairy farms of Prince Edward Island (PE), New Brunswick (NB), Nova Scotia (NS), Ontario (ON), Saskatchewan (SK), Quebec (QC), Manitoba (MB), and Alberta (AB). The primary objective of the project was to obtain reasonably valid estimates for herd-level prevalence of infection with four pathogens of interest (BLV, Bovine Viral Diarrhea Virus, *Mycobacterium avium* subspecies *paratuberculosis*, and *Neospora caninum*) in all of the participant provinces. British Columbia (BC) did not participate in the survey, and Ontario did not administer the questionnaire due to logistical reasons (e.g. funding, coordination, timing). Therefore, those provinces were not included in the present risk-factor study. A political map of

Canada displaying its provinces and territories is presented in Appendix A. The following subsections provide a summary of the sampling and testing protocols.

2.3.1 Herd and animal selection

A stratified two-stage random sampling procedure was applied. Sample size was first based on the calculations carried out for the Atlantic provinces, and then adjusted for the other provinces with respect to the number of available herds, herd size, budget, and other logistics (VanLeeuwen et al., 2001; Tiwari et al., 2009). The minimum number of cows required from each herd to result in a reasonable estimate of within-herd prevalence was obtained based on the following assumptions: an average herd size of 45 lactating cows, within-herd prevalence of 0.05, and confidence level of 0.95. Therefore, approximately 30 cows per herd were needed. In herds with less than 30 cows, all cows were bled and tested. The minimum number of herds required from each province to get reasonably valid estimates for herd-level prevalence of the infection in all of the participant provinces was then calculated based on the following assumptions: an expected herd-level prevalence of 0.70, acceptable relative error of 0.10, and confidence level of 0.95.

2.3.2 Sample collection

During the summer of 1998 in Atlantic Canada (PE,NB, and NS), participating dairy herds were randomly selected, using computer-generated random numbers, from all herds on monthly milk testing (67% of all dairy herds in Canada) through the regional dairy herd improvement (DHI) organization until 90 herds were recruited, 30 herds from each province. These herds met the other herd-level inclusion criteria, including willingness to provide cattle for blood samplings, allowing the blood to be tested for

antibodies, and releasing DHI data to the research team. Subsequently, similar herd-level inclusion criteria and sampling procedures were used to recruit 44, 75, 40, and 66 herds from the provinces of SK, QC, MB, and AB in 2001, 2002, 2002, and 2003, respectively (overall, 315 herds were tested for BLV antibodies). Using computer-generated random numbers, a median of 30 cows (range: 9 – 45) were randomly selected for blood collection from every herd.

2.3.3 Laboratory testing

Within 24 hours after collection, the blood samples were centrifuged, sera were harvested, and stored at -20^{oC} until all of the samples for that province were collected and prepared for testing. The test used for detecting BLV antibodies was an ELISA (IDEXX Corporation, Westbrook, ME, USA; sensitivity = 0.985, and specificity = 0.999). The testing for all provinces except QC was conducted at the national BLV testing laboratory of the Canadian Food Inspection Agency in PE, and the BIOVET Inc. laboratory in QC was utilized for the dairy farms from QC, as requested by the director of the QC portion of the study. A cow was considered to be infected with BLV if the serum-to-positive ratio was ≥ 0.5, as recommended by the manufacturer of the test kit. Every herd with at least one infected animal was defined as being positive.

2.3.4 Data collection and management

Herd-level demographic and management data related to the four mentioned production limiting diseases were collected through personal interviews with owners or managers at each farm. Only questions addressing issues which were hypothesized to be related to BLV seropositivity, such as “transmission of disease through blood”, were

extracted and included in the final data set. Examples of the question details can be found elsewhere (Tiwari et al., 2009).

Additional variables were generated to enable more complete risk factor analysis. The **region** where herds were located was defined as a dichotomous variable: east (PE, NB, NS and QC) versus west (SK, MB, and AB). **Contact index** was generated as a dichotomous variable such that a herd was classified as positive if, over the past five years, any dairy cattle in the herd had contact with cattle from another herd(s) through at least one of the following routes: 1) shared pastures, 2) contract raising of young stock, 3) lending cows/bulls, and 4) borrowing cows/bulls. The **median age** of animals in each herd was the third derived variable of interest. Age of the animals (in months) was retrieved from the DHI database for each herd. Descriptions of all independent variables are presented in Table 2.2.

2.3.5 Statistical analyses

The data were analysed as follows using Stata 13.1 (StataCorp, College Station, Texas, USA). Descriptive statistics (e.g. means, standard deviations, medians, proportions) were calculated for all independent variables to describe the population and to assist in the subsequent modeling process.

Because 59 herds were BLV-negative, a zero-inflated negative binomial (ZINB) regression (Dohoo et al., 2009; Hilbe, 2011) was applied to determine the univariable associations between each independent variable and the proportion of BLV-seropositive cattle on each farm. ZINB models deal with an excessive number of zero counts by simultaneously fitting both binary (usually logistic regression) and count (negative binomial) models. The outcome in the negative binomial (count) part of the ZINB model

was converted from the number of BLV-seropositive cows to the proportion of seropositive cows in a herd (seroprevalence) by adding the number of tested cows in each herd as an exposure term to the models. The association between an independent variable and this outcome was described in terms of an estimated coefficient and its corresponding confidence interval, and of a prevalence ratio in the final multivariable ZINB model. The outcome in the logistic part of the ZINB model was the odds of a zero count or being uninfected; therefore, coefficients have an opposite interpretation to what would be expected in an ordinary logistic regression model. Region was forced into all of the analyses as a potential confounder.

Those independent variables for which the P-value was ≤ 0.15 in either part of the univariable analysis were retained for inclusion in the multivariable model. A backward-elimination strategy was used to build the multivariable model, with a P-value of less than 0.05 (two-tailed) to retain variables. Variable selection was made considering: 1) a theoretical causal web depicting possible relationships among the independent variables and the outcome (Figure 2.1); 2) P-values of the variables in either part of individual ZINB regressions; and 3) correlations among independent variables. All potential two-way interactions between the variables in the multivariable model were assessed, and significant ($P < 0.05$) interactions were retained in the final model.

Finally, in order to assess the predictive ability of the final ZINB model, observed and predicted probabilities of the number of BLV infected animals in the herds were generated and compared. In addition, the Vuong test was applied to compare the goodness-of-fit of the ZINB model versus an ordinary negative binomial regression model containing the same variables.

2.4 Results

Over 95% of the randomly selected herds agreed to participate in the blood sampling and met the inclusion criteria. Complete questionnaires were available for 272 farms, with 59 of those being BLV negative. Table 2.1 has summarized distribution and descriptive statistics for the 272 selected herds and a frequency distribution of the within-herd seroprevalence is presented in Figure 2.2. Thirty positive herds had a within-herd prevalence of less than 10% while seven herds had more than 90% of their animals infected. Based on the univariable analyses, four independent variables (bolded in Table 2.2) were retained for the multivariable modeling.

Of 272 farms, 235 were included in the final modeling process due to no missing data. In the negative binomial part of the final ZINB model, representing factors associated with spread of infection within the farms, herds with clinical cases of leukosis over the last 12 months prior to sampling had a 1.69 times higher within-herd seroprevalence ($P = 0.001$) compared to the herds without such history. Purchasing animals without confirmation of freedom from BLV infection in the last 5 years was also associated with a larger proportion of positive animals ($P < 0.001$) than farms not purchasing cows or farms purchasing cows but only if BLV-negative. Among the two-way interactions, the interaction between "Region" and "Changing gloves for pregnancy check" was found to be statistically significant in the count part of the model ($P = 0.002$); changing gloves between animals in the eastern region was associated with a higher proportion of infection compared to the other combinations. In western provinces, changing gloves was not statistically associated with the proportion of infection within the herds (Table 2.3).

In the logistic part of the model, representing factors associated with spread of infection between the farms, herds in the western region had approximately 3 times higher odds of being infected as compared with their eastern counterparts ($P = 0.006$). Not purchasing cows was positively associated with the odds of herds being free of BLV; farms not purchasing cows in the last 5 years had 6.5 times lower odds of being infected ($P < 0.001$) compared with the farms purchasing cattle without a BLV test requirement.

As illustrated in Figure 2.3, observed and predictive proportion of infected cattle within the herds, based on the ZINB model, were reasonably compatible. Moreover, the Vuong test produced a highly significant P-value of 0.0002, indicating that the ZINB model was clearly superior to a non-inflated (ordinary) negative binomial model.

2.5 Discussion

This study evaluated a wide range of potential herd-level risk factors for BLV in dairy farms from across Canada for the first time. Applying a zero-inflation part to the model demands the possibility of two mechanisms for producing zero counts, other than having extra zero counts (Dohoo et al., 2009; Hilbe, 2011). In our study, we did have these two known mechanisms: 1) a number of the herds (54/235) were defined as negative in the final model based on their serological results and could have been truly free from BLV (certain zeroes in the logistic part); 2) negative herds might theoretically have been positive but we were not able to detect those because of different reasons, including our sampling method and imperfect laboratory tests; therefore, these herds were incorporated in the count part of the model.

The results clearly indicated a high prevalence of BLV infection across the seven study provinces in Canada at the time of sample collection, and this prevalence likely is

still increasing. For instance, in PE, herd-level prevalence of BLV was 49.2% in 1989 (Richardson and Macaulay, 1992), increased to 63.3% in 2001 (VanLeeuwen et al., 2001), and is currently at 90% based on a survey of bulk-tank milk in all dairy herds in the province completed in 2013 (Chapter 5).

Western Canada had a higher seroprevalence of BLV compared to Eastern Canada, which could partly be due to the presence of larger herds in this region. In larger herds, there might be an increased chance of animal exposure to the virus, especially through haematogenous modes of transmission, such as reusing needles for injections, and more intensive reproductive and vaccination programs. The exact number of cattle in the herds was not available; hence, we used the number of registered cows as an indirect surrogate for herd size, which was found to be not associated with the BLV-seropositivity. Other researchers could not find any significant effect of herd size on the prevalence of BLV (Sargeant et al., 1997a; Kobayashi et al., 2010; Murakami et al., 2011). In a Danish study (Gottschau et al., 1990), herd-level prevalence of BLV increased as herd size (in a categorical order) increased. In contrast, when a herd was infected, the proportion of positively tested cattle within that herd decreased with increasing herd size. It should be noted that the herd-level prevalence of EBL in Denmark at the beginning of the study (1970) was very low (<0.3%), which is far less than the previously mentioned studies.

Purchasing cows without knowledge of their infection status, not only for BLV but also for other important contagious diseases of cattle is a recognized risk factor for transmission between herds (Casal et al., 1990; Radostits et al., 2006). Purchasing infected animals would allow these individuals to act as a source for the introduction of

the disease agent to non-infected herds, and because infection is permanent this provides a continuous source for spreading BLV within infected herds. In this study, only 65 farms (25%) had not purchased any cows over the specified period of time, and among the farms with a purchasing history, the majority of them (88%) had not tested their purchased animals for BLV. In order to control a contagious disease such as BLV, producers should either raise their own replacements or buy test-negative animals. Veterinarians should be recommending to their clients the testing of purchased animals for BLV, along with other important infectious diseases.

The association between changing rectal gloves for pregnancy check between animals and seroprevalence of BLV depended on the region in which the herds were located; changing gloves between animals was not statistically associated with the proportion of infected animals in western Canada. In the eastern herds, changing gloves between animals was associated with a greater proportion of positive animals than not changing gloves between animals. The latter finding was contrary to what would be expected, given the current understanding of the role of rectal examination in spreading BLV infection between animals in a herd (Divers et al., 1995). Rectal examination is a potential route of transmission for BLV, but transmission by rectal gloves is also related to other factors such as number of palpations with one common glove, level of contamination of the glove with infected blood lymphocytes, and age of the animals (Radostits et al., 2006). Changing rectal gloves between cows is certainly something that veterinarians can employ for preventing the spread of some infectious diseases, such as BLV, between cows. It is also possible that highly infected eastern herds with known status of the infection were using this strategy to attempt to prevent further spread, or that

there are differences in perceived importance of the disease or employed control measures between the two regions.

The present study did not find significant associations between BLV seropositivity and some well-described risk factors for BLV involving transmission through infected blood, such as multiple-use of needles. One likely explanation for this could be the possible change in management behaviour of the farmers subsequent to identifying clinical cases of BLV-related diseases in their herds (e.g. lymphoma). For instance, we found a significant association between having clinical cases of BLV over the year prior to our study (Table 2.2, which was associated with BLV prevalence in our model as well) and using single-use needles in every injection (results not shown here), suggesting that those herds which applied single-use needles were more likely to have clinical cases. However, after having a clinical case of BLV, farmers might have modified their BLV-control strategies towards posing a lower risk of infection transmission at the time of this study. Therefore, even with applying recommended strategies for controlling the disease, they still demonstrated a high prevalence of infection at the time of completing the questionnaires. This issue is clearly driven by the inherent limitation of cross-sectional studies (reverse causation); such studies are good at establishing association, but poor for ascertaining causality. Reports have also suggested that the importance of haematogenous transmission is variable and may depend on the frequency and the nature of exposure (Gutiérrez et al., 2011; Erskine et al., 2012b). Future prospective studies measuring both management activities and BLV status would be useful for quantifying the risk associated with management factors.

Using gouge dehorning has been identified as another important risk factor in transmitting BLV (DiGiacomo et al., 1985; Radostits et al., 2006; Kobayashi et al., 2010; Erskine et al., 2012b). In the univariable analysis, application of techniques other than gouge dehorners for dehorning was associated with a decreased risk of BLV-positive animals. However, this association was not statistically significant in the final model.

Using pooled milk to feed the calves, having contact with other animals and herds in any of the ways as defined in the "contact index", and having long waiting times before separation of newborn calves from their dams in maternity pens were among other management factors which were found to be not statistically associated with increased prevalence of BLV in the univariable analyses ($p > 0.15$).

Of the 272 herds under study, 235 were included in the final multivariable modeling process. The incomplete information from 37 farms might be attributable to the exhaustive structure of the questionnaire evaluating the four production limiting diseases at the same time. The loss of these herds may have introduced a bias to the results; however, similar distribution of the within-herd prevalence and other descriptive statistics between the 235 and 37 herds suggested that this potential bias was unlikely to be substantial. For instance, BLV prevalence for the final 235 herds was 77%, which was very close to the prevalence for the 272 herds (78.3%).

Although there is always a possibility for misclassification bias in seroprevalence studies, this issue would not substantively affect our analyses for the following reasons: 1) ELISA tests for detecting antibodies against BLV are highly reliable; not only due to very high sensitivity and specificity of the test (nearly perfect), but also because of the permanent nature of BLV infection and relatively stable antibody response (Radostits et

al., 2006); 2) reasonable numbers of animals sampled from each herd led to high herd sensitivities in most of our herds, and only a few herds (5/272) had herd sensitivities less than 0.90; and 3) we applied a ZINB model methodology, which effectively deals with the two scenarios mentioned earlier (i.e. certain and potentially negative herds). Overall, we believe the effect of potential misclassification would be minimal on the presented associations in this study.

2.5.1 Conclusions

The Canadian dairy industry requires a more serious response to its high BLV prevalence. In general, moving towards closed herds in the cattle industry will be an efficient way to control different contagious diseases, wherever possible. National, regional, and herd-specific BLV control programs should not only be focused on inhibiting virus transmission between herds by purchasing BLV-infected replacement animals, but also focused on decreasing BLV spread between cows (within herds) particularly in herds with high prevalence of BLV infection. Additional research is required to establish the relative risk associated with various methods of within herd spread. Applying proper count data models is recommended to extract all of the available information in different data sets on risk factors for infectious diseases of dairy cattle.

2.6 References

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Table 2.1. Distribution of total and bovine leukemia virus (BLV)-seropositive dairy herds which had completed questionnaires in seven Canadian provinces during 1998-2003.

Region	Province	Herds		Number of tested cattle in the herds			Median prevalence in infected herds
		n	Infected [a](%)[b]	Min	Median	Max	
East	Prince Edward Island	30	19 (63.3)	18	30	34	16.7
	Nova Scotia	30	23 (76.7)	9	30	31	13.8
	New Brunswick	30	21 (70.0)	21	30	31	42.8
	Quebec	34	21 (61.8)	20	20	20	35.0
West	Manitoba	39	37 (94.9)	20	30	32	63.3
	Saskatchewan	43	37 (86.0)	31	40	45	43.9
	Alberta	66	55 (83.3)	30	37	37	35.1
Total	-	272	213 (78.3)	9	30	45	36.7

[a] Herds having at least one positive animal were considered infected.
[b] % is the number of infected herds divided by the total number of tested herds, by province.

Table 2.2. Distribution of the variables of interest and results of univariable associations between each variable and bovine leukemia virus (BLV) infection in 272 Canadian dairy herds during 1998-2003[a].

Variables of interest with their corresponding levels	n [b]	Count part	Binary part
		β (95% CI)[c]	β (95% CI)[c]
1. Region [d]	272		
Eastern provinces	124	-	-
Western provinces	148	0.36 (0.13; 0.59)	-1.12 (-1.83; -0.42)
2. Number of registered cows in the herd	238		
<50	111	-	-
50-100	94	-0.16 (-0.43; 0.12)	-0.41 (-1.19; 0.38)
>100	33	-0.15 (-0.52; 0.23)	-1.26 (-3.15; 0.62)
3. Median age of tested animals within each herd (months)	247		
<40	83	-	-
40-50	105	-0.08 (-0.35; 0.19)	0.04 (-0.85; 0.93)
>50	59	-0.01 (-0.34; 0.31)	0.35 (-0.61; 1.31)
4. Having clinical cases of leukosis in the last year (before testing) [d]	238		
No	211	-	-
Yes	27	0.43 (0.08; 0.77)	-1.85 (-4.25; 0.54)
5. Purchasing cows and their BLV status requirements over the last 5 years [d]	256		
Purchased and no testing needed	168	-	-
Purchased and negative status needed	23	-1.05 (-1.57; -0.53)	0.94 (-0.49; 2.37)
No purchases	65	-0.27 (-0.55; 0.01)	1.86 (1.05; 2.68)
6. Using new needles for every injection	253		
No	103	-	-
No, but disinfecting between animals	58	0.12 (-0.17; 0.42)	0.15 (-0.77; 1.08)
Yes	92	0.28 (-0.02; 0.58)	-0.31 (-1.19; 0.57)
7. Using new syringes for every injection	249		
No	153	-	-
Disinfecting between animals or using new syringes	96	-0.06 (-0.26; 0.15)	-0.07 (-0.71; 0.56)
8. Method of dehorning animals	255		
Cutting without disinfection between animals	131	-	-
Cutting with disinfection between animals	39	-0.05 (-0.39; 0.29)	-0.49 (-1.85; 0.86)
Non-cutting methods	85	-0.16 (-0.52; 0.19)	0.52 (-0.47; 1.51)
9. Disinfecting hoof trimming equipment between animals	249		
No	225	-	-
Yes	24	0.09 (-0.33; 0.51)	0.29 (-0.81; 1.39)
10. Disinfecting instruments used for extra teat removal between animals	253		
No	90	-	-
Yes	118	0.007 (-0.26; 0.28)	0.22 (-0.71; 1.15)
Not applicable	45	-0.11 (-0.45; 0.23)	0.34 (-0.74; 1.42)
11. Changing gloves used for pregnancy check between cows [e]	253		
No	187	-	-
Yes	66	0.29 (-0.02; 0.61)	-0.18 (-1.01; -0.65)
12. Changing gloves used for artificial insemination	250		
No	40	-	-
Yes	210	0.09 (-0.23; 0.41)	-0.32 (-1.26; 0.63)
13. Using pooled milk from all cows to feed the calves	255		
No	64	-	-
Yes	191	0.02 (-0.24; 0.28)	0.44 (-0.45; 1.32)
14. Time of separating newborn calves from their dams (hours)	250		
<6	152	-	-
6-12	58	0.17 (-0.11; 0.45)	0.21 (-0.63; 1.05)
>12	40	0.23 (-0.11; 0.55)	-0.05 (-1.02; 0.93)
15. Contact index [f]	256		
No	162	-	-
Yes	94	0.08 (-0.16; 0.31)	0.02 (-0.70; 0.74)

[a] Based on a zero-inflated negative binomial regression analysis for each variable in both parts of the model at the same time and accounting for the number of tested cows in the herds, and region.
[b] Total and subgroup number of herds for each factor.
[c] β is the coefficient of each explanatory variable with the corresponding 95% confidence interval.
[d] P≤0.15 in both parts, so succeeded to both parts of the multivariable model.
[e] P≤0.15 in only count part of the model, so succeeded to the count part of the multivariable model.
[f] "Contact index" was defined as a dichotomous variable such that a herd was classified as positive if, over the past five years, any dairy cattle in the herd had contact with cattle from another herd(s) through at least one of the following routes: shared pastures, contract raising of young stock, and lending or borrowing cows and bulls.

Table 2.3. Output of the final zero-inflated negative binomial (ZINB) model assessing the risk factors of bovine leukemia virus (BLV) infection in 235 Canadian dairy farms during 1998-2003.

Count part (Negative Binomial)	PR[a]	SE	95% CI	P-value
1. Having clinical cases of leukosis over the last 1 year				0.001
No	-[b]	-	-	
Yes	1.69	0.28	1.22-2.33	
2. Purchasing cows and their BLV status requirement over the last 5 years				<0.001[c]
Purchased and no testing needed	-	-	-	
Purchased and negative status needed	0.33	0.08	0.19-0.55	
No purchases	0.83	0.12	0.62-1.19	
3. Region * Changing gloves used for pregnancy check between animals [d]				0.002[c]
East * no	-	-	-	
East * yes	1.87	0.34	1.31-2.69	
West * no	1.70	0.26	1.26-2.29	
West * yes	1.50	0.50	0.78-2.88	
Binary part (Logistic)	**OR[e]**	**SE**	**95% CI**	**P-value**
1. Region				0.006
Eastern provinces	-	-	-	
Western provinces	0.31	0.13	0.14-0.71	
2. Purchasing cows and their BLV status requirement over the last 5 years				<0.001[c]
Purchased and no testing needed	-	-	-	
Purchased and negative status needed	2.54	1.85	0.61-10.61	
No purchases	6.51	2.73	2.86-14.82	

[a] Prevalence Ratio (seroprevalence of BLV infection in a subgroup of herds divided by that in the corresponding reference subgroup of herds).
[b] All reference categories displayed by '-' sign.
[c] Overall P-value for the factor's effect.
[d] Interaction term between region and changing rectal gloves, which was significant overall (P = 0.002).
[e] Odds Ratio (please note that the interpretation of OR in the logistic part of a ZINB model is opposite to an ordinary logistic model).

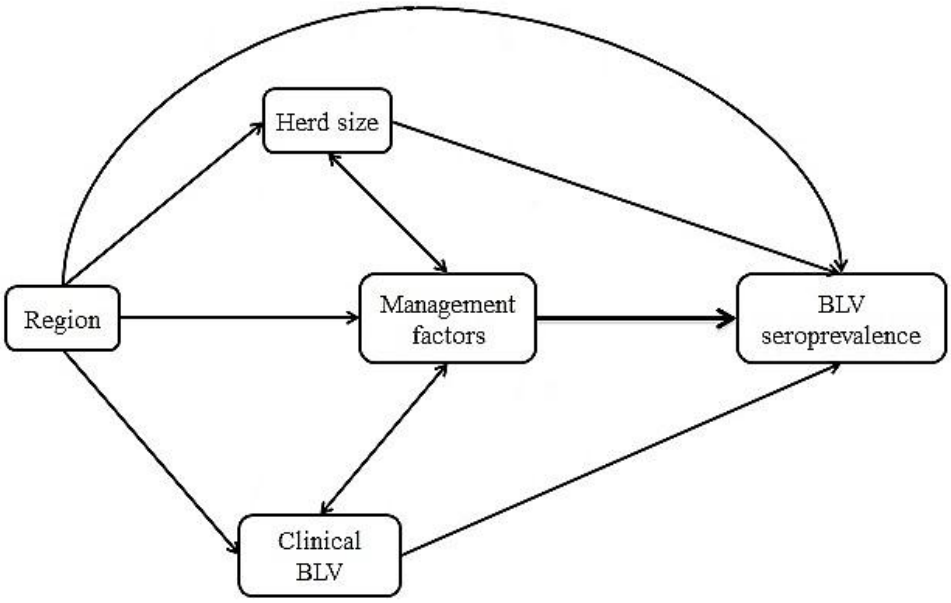

Figure 2.1. A theoretical causal web for seroprevalence of bovine leukemia virus (BLV) infection in 272 Canadian dairy farms during 1998-2003. To keep the web as simple as possible, all management factors were considered as a block, including “purchasing cows”, “median age”, “using new needles and syringes for every injection”, “methods of dehorning”, “disinfecting hoof trimming and extra teat removal tools between cows”, “changing gloves for pregnancy check and artificial insemination between cows”, “Using pooled milk from all cows to feed the calves”, “Time of separating newborn calves from their dams”, and “contact index”. “Clinical BLV” was defined as “having clinical cases of leukosis in the last year before testing”.

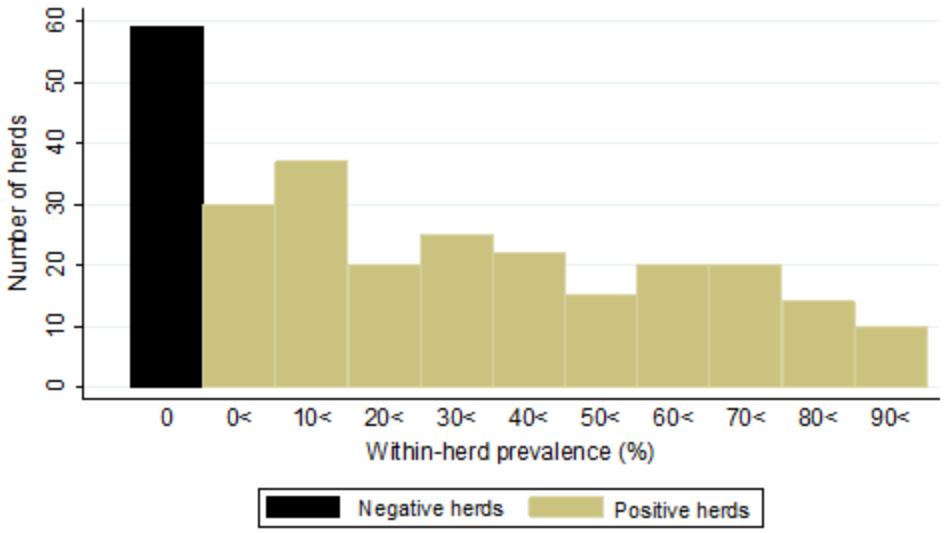

Figure 2.2. Frequency distribution of within-herd seroprevalence of bovine leukemia virus (BLV) infection in 272 Canadian dairy farms during 1998-2003.

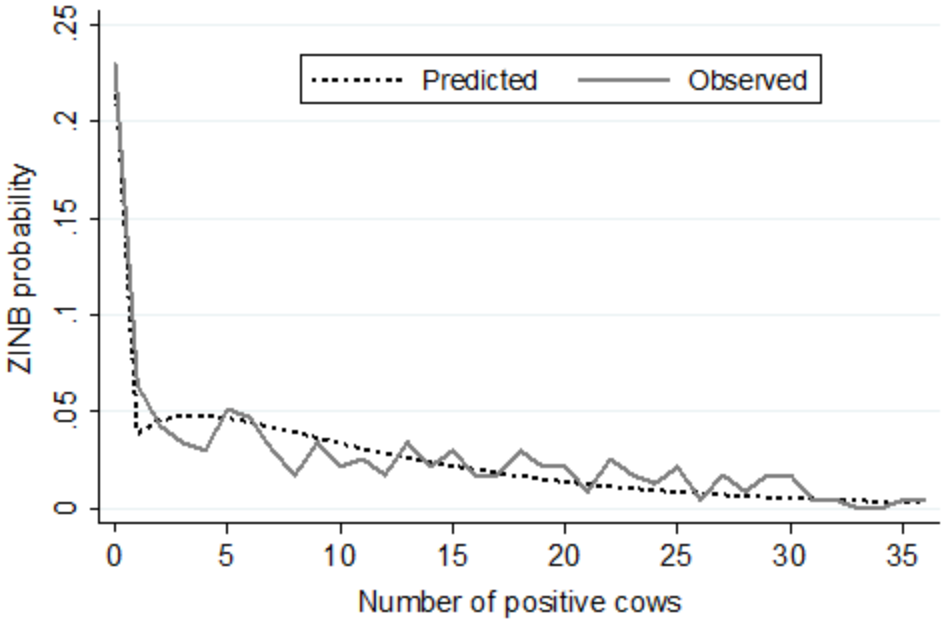

Figure 2.3. Observed and predicted probability distributions for the number of positive animals within the herds, based on the final zero-inflated negative binomial (ZINB) model in 235 Canadian dairy farms. X-axis: range for the number of positive cows in the herds; Y-axis: the results from two different ZINB probability equations (or proportion of observations) (the original and after fitting the final model), when using each X as an input.

CHAPTER 3

LIFETIME EFFECTS OF INFECTION WITH BOVINE LEUKEMIA VIRUS ON MILK PRODUCTION AND LONGEVITY OF CANADIAN DAIRY COWS

3.1 Abstract

Enzootic bovine leukosis (EBL) is an economically important disease of dairy cattle caused by bovine leukemia virus (BLV). The economic impacts of the infection have been debated in the literature. The present study was conducted to determine the lifetime effects of BLV infection on milk production and longevity of dairy cows in Canada.

The data were aggregated from a combination of two data sets: 1) BLV serum ELISA test results from Canada-wide surveys of production limiting diseases which took place between 1998 and 2003 in 8 provinces, and 2) longitudinal production data for all cows in the former study, extracted from the Canadian dairy herd improvement database. All participant cows had been culled or died by the onset of this study. A historical cohort study was designed, including cows which tested positive to BLV-antibodies in their first lactation (positive cohort, n = 1858) and cows which tested negative in their second or later lactations (negative cohort, n = 2194). To assess the impacts of infection with BLV (X) on longevity (Y_1: the number of lifetime lactations), a discrete-time survival analysis was carried out. The effect of BLV on the lifetime milk production (Y_2: the sum of all life 305-day milk production) was evaluated using a multilevel linear regression model.

Overall, 4052 cows from 348 herds met the eligibility criteria and were enrolled in the study. In the longevity model, the interaction term between time (the lactation number) and BLV-status was significant. Cows which were positive to BLV had consistently greater probabilities of being culled (or dying) than the negative cows. In the milk production model, the interaction term between BLV-status and longevity of the cows was highly significant; indicating that lifetime BLV effects on the total milk

production was dependent on the lactation in which the study cows were culled or died. Infected cows with 2 and 3 lactations showed significantly lower life milk productions [-2554 kg (-3609 to -1500) and -1171 kg (-2051 to -292), respectively] compared with their negative counterparts with 2 and 3 lactations. Overall, as the cows lived longer (> 3 lactations), the differences in life milk production between the two cohorts were no longer significant. With the high prevalence of BLV infection in Canadian dairy cows and its detrimental economic impacts, pursuing a broad-based control program in Canada is necessary.

3.2 Introduction

Enzootic bovine leukosis (EBL) is an economically important disease of dairy cattle across the world. The causative agent of EBL is a retrovirus, bovine leukemia virus (BLV). The virus is transmitted through infected blood lymphocytes (Gillet et al., 2013). Once cattle become infected with BLV, they remain infected for life and generate a continuous antibody response (Monti et al., 2007; Kobayashi et al., 2010). Clinical signs of the disease are not displayed by most infected cattle. Persistent lymphocytosis (PL) will occur in approximately 30% of infected cattle and fewer than 5% will eventually develop malignant lymphoma (Radostits et al., 2006).

The prevalence of BLV infection in North America has been high and appears to have a rising trend (Bartlett et al., 2014; Chapter 2). For instance, in the Maritime region of Canada, herd-level prevalence of BLV infection from 70% in 1998 (VanLeeuwen et al., 2001) reached to over 90% in 2013 (Chapter 5). In the United States, as a part of the 2007 national dairy study (APHIS-USDA report, 2008), 83.9% of the tested herds were found to be positive.

Measured at the herd level, the direct production losses from EBL in the Canadian Maritimes were conservatively estimated at $806 per year in an average 50-cow herd (Chi et al., 2002). This did not include costs associated with lost sales of genetically superior purebred cattle which are likely more substantial than the direct production impacts. In another study, economic loss per case of lymphoma was estimated to be $412 (Rhodes et al., 2003). With respect to the economic impacts of BLV infection, the most significant losses attributed to the disease are: premature culling, death, and condemnation of carcasses at slaughter due to lymphoma, production loss, lower reproductive efficiency, impaired immune function, as well as trade restrictions imposed on infected cattle and their products (Sandev et al., 2000; Bartlett et al., 2014). However, reports on production and longevity effects of subclinical BLV in dairy cattle have been quite controversial in the literature. A number of studies could not demonstrate any statistically significant association between BLV infection and milk production in dairy cows and herds (Landston et al., 1978; Huber et al., 1981; Brenner et al., 1989; Da et al., 1993; Tiwari et al., 2007; Sorge et al., 2011). In contrast, detrimental effects of BLV infection on production have been documented by others (Emanuelson et al., 1992; Sargeant et al., 1997; D'Angelino et al., 1998; Ott et al., 2003; Erskine et al., 2012). A few studies failed to find any statistically significant association between BLV infection and the survival of dairy cows (Huber et al., 1981; Tiwari et al., 2005). On the other hand, others have reported negative effects of BLV infection on the longevity of dairy cows (Pollari et al., 1992; Emanuelson et al., 1992; Bartlett et al., 2013). Moreover, the majority of the preceding studies were not able to provide rigorous evidence for establishing strong associations between BLV infection and production measures (or

longevity) of dairy cows. Some of the likely reasons for obtaining such inconsistent findings were: 1) the cross-sectional nature of these studies; 2) limited numbers of study cows or herds; 3) in some studies, poor control for potential confounders and application of unsuitable statistical analyses; and 4) the use of only production data in the lactations in which BLV testing was performed (therefore, not accounting for potential seroconversions and progression of the infection pathology). In addition, there has not been any study investigating the lifetime impacts of BLV infection, which could economically be more relevant with regards to the chronic nature and gradual progression of the infection. Therefore, this historical cohort study was conducted to determine the effects of BLV infection on 1) lifetime milk production, and 2) longevity of dairy cows in Canada.

3.3 Materials and methods

In order to assess the lifetime effects of BLV infection on cow longevity and milk production, the following steps were taken: 1) generating a master data set by merging available BLV test results (from a previous study) with longitudinal lifetime production data for the study cows; 2) defining a pool of eligible cows for selecting two comparable cohorts of cows (negative and positive to BLV, in a historical cohort setting); and 3) longitudinally evaluating the two cohorts of cows with respect to the longevity and milk production measures using multilevel regression analyses. The following subsections elaborate on the details of the 3 steps, respectively.

3.3.1 Data collection and management

The master data set used in this study was generated via combining two data sets, the historic BLV test results and the longitudinal production data. The BLV test results

were extracted from the Canada-wide surveys of production limiting diseases (Chapter 2) that took place between 1998 and 2003 on randomly selected dairy farms in 8 (out of 10) provinces of Canada (i.e. source population), including Prince Edward Island (PE), New Brunswick (NB), Nova Scotia (NS), Quebec (QC), Ontario (ON), Manitoba (MB), Saskatchewan (SK), and Alberta (AB). One of the objectives of that project was to obtain estimates for the prevalence of infection with BLV in all participant provinces. Herd and animal selections in the surveys were based on a stratified two-stage random sampling procedure. From every randomly selected herd (n = 364), a median of 30 cows (range: 9–45) at different lactations were randomly selected for blood collection. The blood samples were tested for BLV antibodies by a commercial serum ELISA (IDEXX Corporation, Westbrook, ME, USA; sensitivity = 0.985 and specificity = 0.999). A cow was considered to be infected with BLV if the serum-to-positive ratio was ≥ 0.5, as recommended by the manufacturer of the test kit. Every herd with at least one infected cow was defined as being positive (VanLeeuwen et al., 2001). Complete details on the surveys, including sample size calculation, sample collection, and laboratory procedures have been published elsewhere (Tiwari et al., 2009; Chapter 2).

In 2013, based on the available unique identification numbers for all participant herds and cows in the former broad surveys, production data (including 305-day milk yield (kg), 305-day fat and protein contents of milk (kg), days-in-milk (DIM), average somatic cell count (SCC)) for all lactations of a cow during her lifetime was extracted from the Canadian dairy herd improvement (DHI) database and combined with the BLV test results. All of the cows had been culled or died by the onset of the present study.

3.3.2 Study design

The eligible pool of cows for inclusion in this historical cohort study (i.e. sampling frame) consisted of Holstein cows (96% of all cows - to eliminate breed as a potential confounder) with complete longitudinal data for lactation measurements (from their first lactation to their culling point, with no missing observations; 90% of all Holsteins). From this pool, two cohorts of cows (negative and positive to BLV) were selected as follows:

- Positive cohort: cows that tested positive to BLV in their first lactation. Thus, this group of cows was considered infected for all of their productive life.
- Negative cohort: cows that tested negative to BLV in their second or later lactations (i.e., disregarding the first lactation test-negative cows). Therefore, this group of cows was considered negative from birth to at least the beginning of their third lactation.

Due to the definition of the negative cohort, only cows with 2 lactations or more were included in the study. The reason for applying the outlined inclusion criteria was to reduce two major potential sources of bias in establishing the associations of interest in the statistical analyses (see section 3.5 for the complete details).

3.3.3 Statistical analyses

All of the statistical analyses were carried out in Stata 14 (StataCorp, College Station, TX, USA).

3.3.3.1 Longevity effects

To assess the effect of infection with BLV (exposure variable, X) on the longevity of study cows, the total number of lactations for each cow was used as a measure of longevity (Y: lifetime lactations). According to a theoretical causal web (Figure 3.1), available variables in the data set, which could potentially have played a role as

confounders regarding the association of interest, were incorporated in the modeling process (clustering effects of herds and provinces). Within-herd prevalence of BLV was included in the modeling because of its interpretation as a contextual effect. Hypothesized intervening variables (such as SCC, 305-day milk, fat, and protein) were excluded from the modeling.

To provide a visual representation for the overall impact of BLV infection on longevity of the study cows, a Kaplan-Meier survival plot was generated. Because the outcome of interest was inherently discrete, a discrete-time survival analysis (Dohoo et al., 2009) was performed as follows: 1) the number of life lactations (Y) was set in a survival-time framework; 100% failure (i.e. culling or death) occurred in all study cows; 2) data for each cow was then organized on a one-observation per lactation basis (survival-time splitting), with failure occurring in her last lifetime lactation; and 3) mixed-effects logistic regression models were built to evaluate the effect of infection with BLV on the failure of cows across all lactations.

3.3.3.2 Lifetime milk production effects

In order to assess the effect of infection with BLV (X) on lifetime milk production of the study cows, the sum of all 305-day milk (kg) produced by each cow during her life was calculated to serve as the outcome of interest (Y: lifetime milk). According to a theoretical causal web (Figure 3.2), available variables in the data set, which could potentially have played a role as confounders regarding the association of interest, were utilized in the modeling process. To evaluate the association of interest, mixed-effects linear regression models were built, incorporating study herds and provinces as random effects. Longevity was included in the models in order to estimate the direct effect of

BLV on the life milk production, also in order to avoid multimodal residual distributions. Hypothesized intervening variables (such as SCC, 305-day milk, fat, and protein) were excluded from the models.

3.4 Results

Overall, the master data set consisted of 45,704 lactations for 10,670 cows (with one BLV test result per cow) from 364 herds in 8 provinces of Canada.

Of 10,670 tested cows in the master dataset, 31.7% (n = 3390) were positive to BLV-antibodies in the ELISA test. Table 3.1 presents the frequency of tested herds and cows in the master data set (source population) and the cohort study by province. Overall, 4052 cows (with 2-13 life lactations) from 348 herds (with an average of 11.6 cows per herd- range: 1-29) met the eligibility criteria and were enrolled in the historical cohort study. The two cohorts of negative and positive to BLV included 2194 and 1858 cows, respectively (45.8% positive). Table 3.2 presents a descriptive summary of the variables investigated in the study.

3.4.1 Longevity effects

Figure 3.3 depicts the Kaplan-Meier survival plot for longevity (lifetime lactations) of the two cohorts of cows. According to the graph, BLV-positive cows show a clear shorter longevity as compared with BLV-negative cows. All positive cows were culled/died prior to their tenth life lactation; only 19 (1%) and 2 (0.1%) positive cows lived to their 8^{th} and 9^{th} lactations, respectively. In the negative cohort, 13.5% of the cows experienced lactations > 7 (8 to 13). Hence, in order to make meaningful comparisons between the two cohorts, the final logistic model was confined to the life lactations < 8 (by censoring lactations > 7 in the study cows- this period is also consistent with the

economic life of dairy cows). The output for the final mixed-effects logistic model is presented in Table 3.3. This model included 14,243 lactations, from the 4052 cows.

Random effects of province explained only 3.5% of the total unexplained variation in the final model, when approximating the lowest-level variance by 3.29 (Dohoo et al., 2009). Variability among herds explained 7.4% of the total variation, suggesting altogether minor clustering effects for cows within the herds and provinces with regard to the longevity. Estimated within-herd prevalence of BLV was positively associated with the longevity of the study cows ($P = 0.002$), indicating that cows from highly infected herds tended to have shorter lives compared to the cows from low-prevalence herds (that is a contextual effect of BLV infection).

In the final model (Table 3.3), the interaction term between time (i.e. lactation number) and BLV status was highly significant ($P < 0.001$), indicating that the effect of BLV infection on the odds of failure (culling or death) was dependent on the lactation under consideration. Therefore, an interaction plot was produced to illustrate the relationship between the predicted probability of culling/death and longevity by BLV status for the study cows, while the within-herd prevalence was held constant at its mean value of 0.36 (Figure 3.4). From the graph, BLV-positive cows had constantly greater probability of being culled (or dying) than BLV-negative cows over the course of the comparison ($P < 0.001$ for all lactations). As the cows lived longer, the difference in the probability of culling between the two cohorts gradually increased (with an exception in the 5^{th} life lactation), from 13.4% at the second lactation to 26.2% at the seventh lactation. On the other hand, the relative impact of BLV in terms of the odds-ratio was largest in the second lactation (Table 3.3).

3.4.2 Lifetime milk production effects

Similar to the longevity analyses, in order to make meaningful comparisons between the two cohorts of cows, and to be consistent with the economic life of dairy cows, lifetime milk production for cows with 2-7 lactations was the outcome in the analysis.

To satisfy the assumptions of mixed-effects linear models, lifetime milk production was square-root transformed and used as the outcome of interest. Within-herd prevalence of BLV (the contextual effect) did not show any significant effects on the outcome; therefore, it was not included in the final model (Table 3.4). The interaction term between BLV-status and longevity of the cows was highly significant ($P < 0.001$), indicating that lifetime effects of BLV infection on the total milk production was dependent on the lactation number in which the study cows were culled/died. This interaction is illustrated in Figure 3.5. As depicted, BLV-positive cows with 2 and 3 lactations (i.e. with short longevity) showed significantly lower life milk productions: (after back-transformation to original scale) -2554 kg (95% CI: -3609 to -1500) and -1171 kg (95% CI: -2051 to -292), respectively, as compared with their negative counterparts with 2 and 3 lactations. As the cows lived longer (> 3 lactations), the differences in life milk production between the two cohorts were no longer substantial, nor statistically significant.

Despite the fact that the random effect of province was statistically significant in the final model, it only explained 5.8% of the total unexplained variation in the model. In contrast, the random effect of herds explained a considerable proportion of the total variation in the model (24.5%). Over 69% of the variation in the lifetime milk production was due to variability among the individual cows.

3.5 Discussion

This was the first time that the lifetime impacts of BLV infection were investigated on a broad scale. Our study provided more rigorous evidence for direct effects of BLV infection on longevity and milk production as compared with the historic studies, because of the following advantages: 1) wide study area (herds and cows from across Canada); 2) a large number of participants (4052 cows from 348 herds); 3) study design (historical cohort), which is the strongest type among observational studies towards establishing causal associations (Dohoo et al., 2009); and 4) including data from all life lactations of the study cows which can reflect the gradual nature and impacts of BLV infection.

Previous studies on production and longevity effects of BLV infection (mentioned in the introduction section) were cross-sectional and incorporated production data restricted to the lactations in which BLV-testing was implemented or a few lactations afterwards. As a result, their findings could have been influenced by two major sources of bias:

1) Positive cows: for most of the BLV-positive cows, it was not clear when exactly they became infected. For instance, assume a cow that was diagnosed positive by a test conducted in her late life lactations (e.g. > 4); this cow could have become infected at any time prior to the testing date, while it was simply regarded as BLV-positive in the analyses. If the cow became infected early in life (i.e. far from the testing date), production effects of the infection could be well reflected by the later lactations. Conversely, if this cow became infected sometime closer to the testing date (e.g. during the fourth lactation or later), the concurrent production data might not truly represent the

impacts of BLV infection because such effects gradually develop over time. In particular, the adverse effects due to developing PL and/or lymphoma have a strong association with reduced production and survival of infected cows (Pollari et al., 1992; Da et al., 1993; Radostits et al., 2006). To address this possible source of bias, we restricted our positive cohort to the cows, which were tested during their first lactation only. Hence, these cows certainly became test-positive early in their life (before or during the first lactation), and their lifetime effects could reflect the true impacts of BLV infection more sensibly during the subsequent lactations.

2) Negative cows: BLV-negative cows could have become infected (seroconverted) at any time after the lactation in which BLV testing was done and therefore introduced a partial misclassification bias. This issue could particularly be concerning when negative cows were tested early in life. In this case, the potential BLV impacts would gradually develop during the consequent life lactations and confound the results. In our study, we restricted the negative cohort to cows that were tested negative at least in their second lactations. Therefore, we should not expect substantial bias due to likely seroconversions in an appreciable fraction of negative cows, because: 1) the probability of developing new infections (i.e. incidence rate, not prevalence) is expected to gradually decline after 4-5 years of age (Radostits et al., 2006; Kale et al., 2007); 2) 55.5 % of our study cows in the negative cohort had been tested during their third or later lactations (thus the likelihood of seroconversion and its later life impacts would be even less important); and 3) even if some new infections occurred following the second lactation (> 4 years of age) in a fraction of negative cows, there should not be prominent effects on the total (lifetime) production because of the gradual progress and chronic nature of BLV

infection. We did not further limit our negative cohort to the cows tested in their third or greater lactations due to loss of many observations (25%) in the study. However, to address any potential concerns, a sensitivity analysis was done in which the negative cohort was restricted to cows that were exclusively tested negative in their third or later lactations; our main conclusions were unaffected (results not shown).

To entirely remove the outlined sources of bias, repeated testing for BLV (to detect any new occurrence of the infection), along with the differential blood cell count (to record any possible change/progress in the course of the infection; e.g. advancing to PL status) during the lifespan of cows would be required. However, additional testing would add substantial practical and cost implications in large field studies such as ours. Pollari et al. (1992) and Da et al. (1993) were the only researchers who implemented repeated testing for BLV and differential blood cell counts over 3- and 6-year periods (respectively) to record potential seroconversions, and progress of the infection. However, their studies were confined to one dairy herd with limited number of cows. Therefore, the generalizability of their findings could be questionable. They reported that the adverse effects of BLV infection were primarily limited to PL cows, which were culled earlier and had reduced milk during their culling lactations.

With respect to the natural history of BLV infection, evaluating its impacts on production parameters in the long-term could be much more enlightening than during only one (concurrent) or a few lactations after testing. Approximately, 30% of the infected cattle develop PL during their life (Schwartz and Levy, 1994). Persistent lymphocytosis without clinical signs can occur earlier in life, but rarely before 2 years of age. Many cows remain in the preclinical stage for years, often for their complete

productive lifetime without any apparent reduction in performance, but lymphoma eventually appears in a proportion of these cows (in 5% of the infected cows). Lymphoma is rarely seen in animals less than 2 years of age and is most common during lactations 2 to 6 (Radostits et al., 2006; Smith, 2009). This period of high risk, during which observing the potential impacts of BLV infection on the performance would be expected, was well covered in the present study.

3.5.1 Longevity effects

Overall, infected cows lived significantly shorter than their negative counterparts in our study, suggesting that infection with BLV could in fact be one of the causes of premature culling (or death) in any age group of cows. This effect was not substantially influenced by the herd of origin (e.g. by herd-level management decisions) because approximately 90% of the unexplained variation in the likelihood of culling was explained by the individual cow variability.

As the study cows aged, the probability of culling remained significantly higher in the positive cohort compared to the negative cohort. This is in agreement with the gradual progress of the infection over time; for instance, because of increasing the number of cows with PL and lymphoma which could lead to a higher culling rate in older infected cows. In this regard, adverse consequences of the infection, such as abnormal immune function and reproductive performance, could play an important role in vulnerability to other infections such as mastitis (Vanleeuwen et al., 2010; Frie and Coussens, 2015). In a large Swedish study, it was suggested that the risk for other infectious diseases of dairy cattle was greater in BLV-positive herds compared to BLV-negative herds (Emanuelson et al., 1992). In the Canadian dairy industry, reproductive disorders, mastitis, and feet and

leg problems have usually been the top three causes for culling and replacements (Canada dairy-info, 2015). Considering its high prevalence in Canada, infection with BLV (particularly in the long-term) could act as a confounder for the effects of the mentioned disorders on culling rate. Culling cows before they reach their maximum genetic potential for production and reproduction is one the most important sources of economic loss in all dairy herds (Chi et al., 2002).

Thurmond et al. (1985) reported longer survival (beyond 3.5 years of age) among antibody-negative cows than among positive cows in a dairy herd. Erskine et al. (2012) found a herd-level negative association between cow age, estimated as the percentage of the herd in the third or greater lactations, and BLV prevalence. Bartlett et al. (2013) investigated the longevity of BLV infected cows in 112 dairy herds from Michigan and showed that infected cows were 23% more likely to be culled or die compared with their negative counterparts. However, they only followed the cows for an average of 597 days after BLV testing and only 48.3% of the cows were culled by the end of study. In contrast, a few studies failed to find any statistically significant association between BLV infection and the survival of dairy cows, although they did not look at lifetime longevity (Huber et al., 1981; Tiwari et al., 2005). An additional caution should be taken when interpreting culling results if the owners received the BLV test-positive results – cows could have been culled just because they were BLV test-positive without any clinical or subclinical manifestation of the infection, especially if a farmer had a small number of test-positive cows and eradication was the farm goal. This could result in some overestimation in the significance of the association of interest (i.e. bias away from the null); however, the magnitude of this potential bias is believed to be low.

3.5.2 Milk production effects

Including the interaction term between BLV infection status and lifetime lactations in the analysis allowed for comparison between the two cohorts of cows at each age group. The quantity of loss in milk production for BLV-positive cows that were culled in their second and third lactations was substantial. One of the possible reasons for this finding could be adverse impacts of advanced BLV infection (e.g. PL stage) on the milk production, or indirectly through compromised immune function (Kabeya et al., 2001; Frie and Coussens, 2015). Hence, the affected cows were presumably removed from the herds due to insufficient milk production or other reasons in early lactations. In addition, it could be assumed that the positive cows that lived longer were mostly among those that did not develop advanced stages of the infection (approximately 70% of infected cows) and/or were genetically superior cows with high potential for milk production. Overall, it appears that subclinical BLV infection did not have a prominent effect on life milk production for the cows that lived their expected economic life (> 5 years of age). The impact of BLV infection on lifetime milk production was clustered within the herds, indicating the actual differences varied among these herds with regards to several factors, which could include genetic value/merit of their cows, nutrition, and other management practices.

If one were to consider a partial budget for total BLV economic impact, the culling of poor milk-producing cows transfers the cost of poor production from the milk production component to the culling component. Therefore, overall survival of BLV-infected dairy cattle may be a more comprehensive measure of the impact of BLV on a dairy herd in that it includes cow removal from the herd for a variety of reasons that may result from hypothesized BLV-altered immune function (Bartlett et al., 2013).

Sorge et al. (2011) could not demonstrate any significant association between BLV infection and milk production of 19,785 cows from 258 herds in Ontario and western Canada. They only evaluated the test-day lactations and combined the suspicious samples with the negative results, which might have caused the non-significant effect. There have also been other studies which could not reveal any significant association between BLV infection and production measures (Brenner et al., 1989; Jacobs et al., 1991; Kale et al., 2007). However, none of these studies looked at lifetime parameters.

Erskine et al. (2012) reported that each 0.1 increase in the proportion of positive cattle in 104 Michigan dairy herds was significantly associated with a 115 kg decrease in 12-month rolling herd average milk yield per cow. Ott et al. (2003), in a large US study, showed that herds with seropositive cows produced 218 kg per cow less milk compared with negative herds. Da et al. (1993) demonstrated that positive herds produced 3% less milk per cow. However, it is notable that the herd-level evaluations are prone to "ecological fallacy"; that is, the results may not be generalizable to the individual cows – some infected cows may be producing more milk than non-infected cows in a positive herd (Jacobs et al., 1991).

In a study limited to one 219-cow dairy herd in the US, it was shown that milk production during the testing lactation in seropositive cows was lower than that in their negative herd mates, after adjustment for genetic potential for milk production in the study cows (Wu et al., 1989). With respect to the importance of the discovered links between genetic composition of cows and vulnerability to BLV infection and its progress rate (Jubb et al., 1993; Fenner et al., 2011; Frie and Coussens, 2015), controlling for

production merits of cows in the analyses could be of particular interest. We, however, did not have access to this information.

Another limitation in most BLV-focused studies (including the present study) is that no information on progress and course of the infection is available. As discussed, 30% of the infected cows will develop PL at some point in their life, which could prominently affect their production (Pollari et al., 1992; Da et al., 1993). Despite being costly and sometimes impractical in field conditions, repeated testing for BLV and lymphocyte counts over the economic life of cows could provide valuable insight into the definitive impacts of the infection. With the high prevalence of BLV infection across Canada (see Chapter 5 for a prevalence update) and its outlined negative economic impacts, pursuing broad-based, efficient control programs in Canada is imperative.

3.5.3 Conclusions

With the chronic nature and gradual progress of BLV infection, evaluating BLV's economic impacts over the lifetime of dairy cows is very informative. The design of the present study (historical cohort) was well suited for describing the potential causal associations between BLV infection and the corresponding lifetime production and longevity effects. Seropositive cows had consistently shorter lifespans compared with their negative counterparts, suggesting that the infection could be one of the main causes of premature culling (or death) at any age groups of cows. Seropositive cows that were culled at early lactations (second and third) exhibited substantially lower milk production than seronegative cows. However, the lifetime milk production in the two cohorts among long-living cows (> 3 lactations) did not show any significant difference. Overall, with

the high prevalence of BLV infection across Canada and its detrimental economic impacts, pursuing broad-based control programs is highly recommended.

3.6 References

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Table 3.1. Frequency distribution of the tested herds and cows for bovine leukemia virus antibodies in source (M) and study (S) populations, from 8 provinces of Canada.

Province	No. of tested herds		No. of tested cows		No. of positive cows		Proportion of positive cows (%)[a]	
	M[b]	S[c]	M	S	M	S	M	S
Prince Edward Island	30	29	805	340	150	42	18.6	12.3
New Brunswick	30	26	791	251	106	23	13.4	9.1
Nova Scotia	30	29	785	327	253	71	32.2	21.7
Quebec	93	87	2285	637	632	349	27.6	54.7
Ontario	31	31	800	281	260	134	32.5	47.6
Manitoba	40	37	1109	563	643	511	57.9	90.7
Saskatchewan	44	44	1641	692	585	249	35.6	35.9
Alberta	66	65	2454	961	761	479	31.0	49.8
Total	364	348	10670	4052	3390	1858	31.7	45.8

[a] Number of positive cows/Number of tested cows
[b] M: in master data set (source population)
[c] S: in study population

Table 3.2. Summary statistics for the variables used in the study on 4052 dairy cows.

Variable[a]	Brief description	Mean	SD	Min	Median	Max
Longevity	Total life lactations: Y_1[b]	4.65	1.87	2	4	13
Milk (kg)	Total life 305-day milk yield:Y_2[c]	38629.23	18940.27	3177	36273	141892
WHP[d] (%)	Within-herd prevalence	36.23	30.71	0	33.33	100
Fat (kg)	Average of all 305-day fat	328.52	61.05	129	328.25	611.50
Protein (kg)	Average of all 305-day protein	292.88	50.15	100	294.50	521
Ln-SCC[e]	Average of all ln-SCC	3.10	1.28	0.10	2.93	8.50

[a] "Fat, Protein, and Ln-SCC" were originally lactation-level variables which have been averaged at the cow level.
[b] The outcome of interest for evaluating the effect of bovine leukemia virus infection on longevity (Y_1).
[c] The outcome of interest for evaluating the effect of bovine leukemia virus infection on lifetime milk production (Y_2).
[d] Estimates of within-herd prevalence of infection with bovine leukemia virus in the study herds.
[e] Average linear score of somatic cell counts was only available for 3654 cows (398 were missing).

Table 3.3. Results of the final mixed-effects logistic model, evaluating the lifetime effects of infection with bovine leukemia virus (BLV) on the log-odds of culling/death for the 4052 study cows.

Variable	Coefficient	SE	95% CI		Odds-ratio
Fixed effects					
Life lactation					
2	-	-	-	-	-
3	1.24	0.12	1.01	1.48	3.47
4	1.79	0.17	1.57	2.02	6.02
5	2.37	0.12	2.14	2.59	10.68
6	2.81	0.12	2.57	3.06	16.71
7	3.03	0.13	2.77	3.29	20.76
BLV * Life lactation [a]					
2	1.52	0.12	1.27	1.76	4.57
3	1.05	0.09	0.86	1.23	2.86
4	1.02	0.09	0.84	1.21	2.79
5	0.83	0.11	0.61	1.05	2.29
6	0.89	0.15	0.59	1.19	2.44
7	1.23	0.27	0.71	1.76	3.44
Within-herd prevalence	0.31	0.10	0.11	0.51	1.36
Constant	-3.23	0.11	-3.44	-3.02	-
Random effect variances					
Province	0.13	0.11	0.02	0.64	-
Herd	0.27	0.07	0.17	0.45	-

[a] Interaction term between BLV infection status and number of life lactations.

Table 3.4. Results of the final mixed-effects linear model, evaluating the lifetime effects of infection with bovine leukemia virus (BLV) on the square-root of total life milk production (kg) for the 4052 study cows.

Variable	Coefficient	SE	95% CI	
Fixed effects				
Life lactation				
2	-	-	-	-
3	26.49	2.14	22.31	30.68
4	52.05	2.08	47.98	56.13
5	74.46	2.06	70.43	78.49
6	98.66	2.09	94.56	102.77
7	121.03	2.04	117.02	125.04
BLV * Life lactation [a]				
2	-10.58	2.17	-14.84	-6.32
3	-3.89	1.48	-6.80	-0.98
4	0.93	1.43	-1.88	3.74
5	1.11	1.52	-1.86	4.08
6	1.72	1.83	-1.87	5.31
7	0.31	2.24	-4.08	4.70
Constant	125.99	2.71	120.68	131.30
Random effect variances				
Province	27.74	17.52	8.04	95.66
Herd	116.14	11.96	94.91	142.12
Cow	331.39	7.71	316.61	346.85

[a] Interaction term between BLV infection status and number of life lactations.

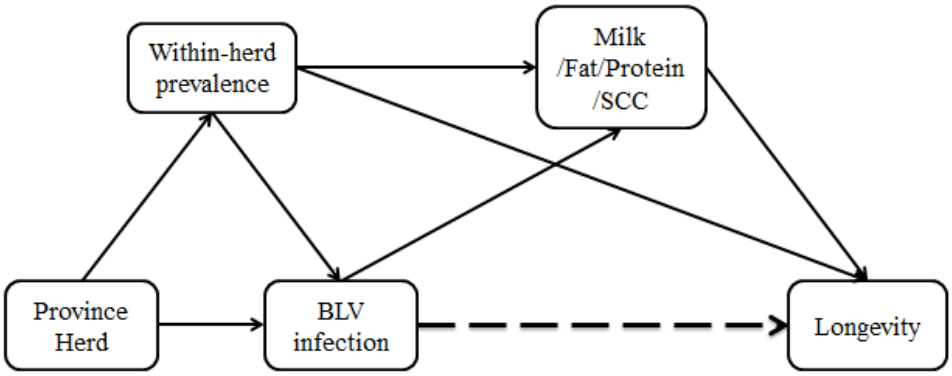

Figure 3.1. A theoretical causal web, illustrating relationships among the potential confounding or intervening variables with respect to the association of interest (dashed arrow) between bovine leukemia virus (BLV) infection (exposure) and longevity (Lifetime lactations). Random effects of "province" and "herd" clustering the study cows have been included. Within-herd prevalence of BLV infection (contextual effect), average lifetime 305-day milk, fat, protein, and average linear score of somatic cell count (SCC) at the cow level (as a block) were considered as well.

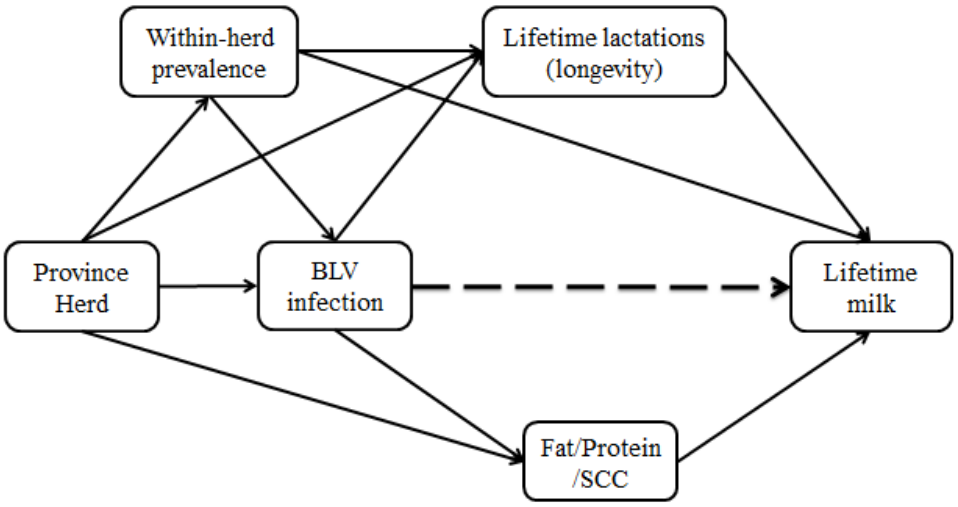

Figure 3.2. A theoretical causal web, illustrating relationships among the potential confounding or intervening variables with respect to the association of interest (dashed arrow) between bovine leukemia virus (BLV) infection (exposure) and lifetime 305-day milk production (Lifetime milk). Random effects of "province" and "herd" clustering the study cows have been included. Within-herd prevalence of BLV infection (contextual effect); the total number of life lactations (longevity); as well as average lifetime 305-day fat, protein, and average linear score of somatic cell count (SCC) at the cow level (as a block) were considered.

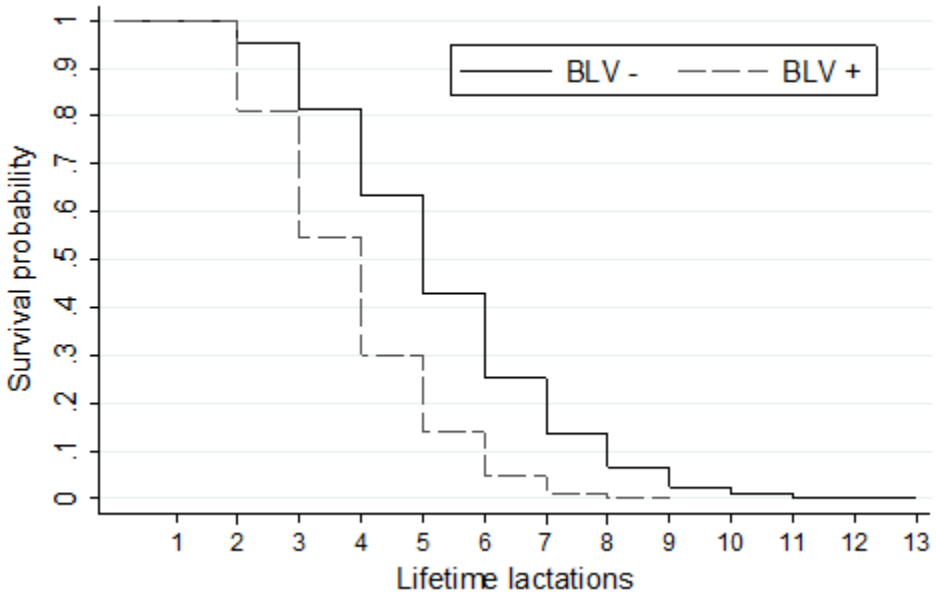

Figure 3.3. Kaplan-Meier survival plot for 4052 study cows, by the two cohorts of test-negative and test-positive cows for bovine leukemia virus (BLV) infection.

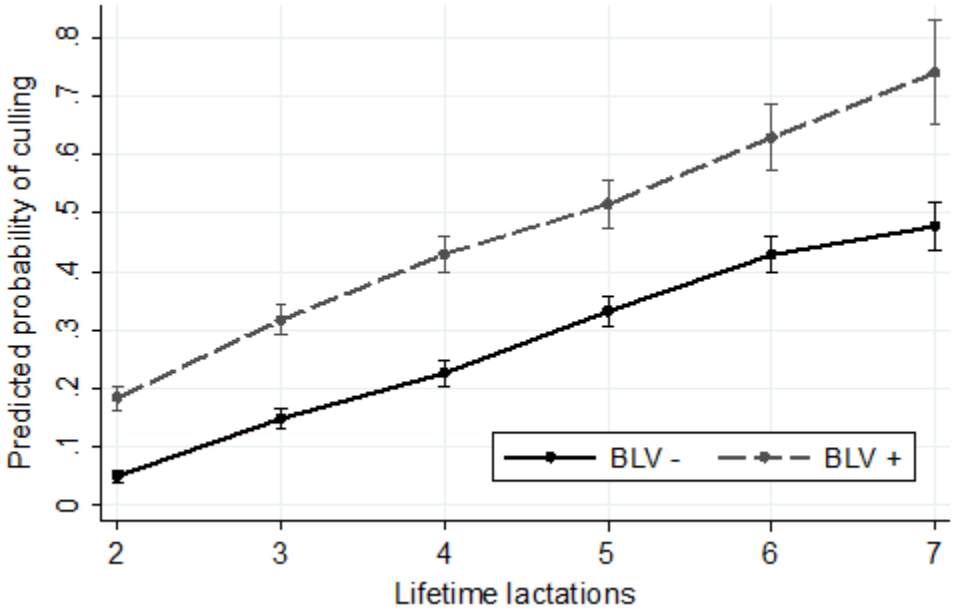

Figure 3.4. Interaction plot illustrating the relationship between the predicted probability of culling/death (Y-axis) and longevity (lifetime lactations; X-axis) by bovine leukemia virus (BLV) infection status, along with the corresponding 95% confidence intervals (spikes) for 4052 study cows.

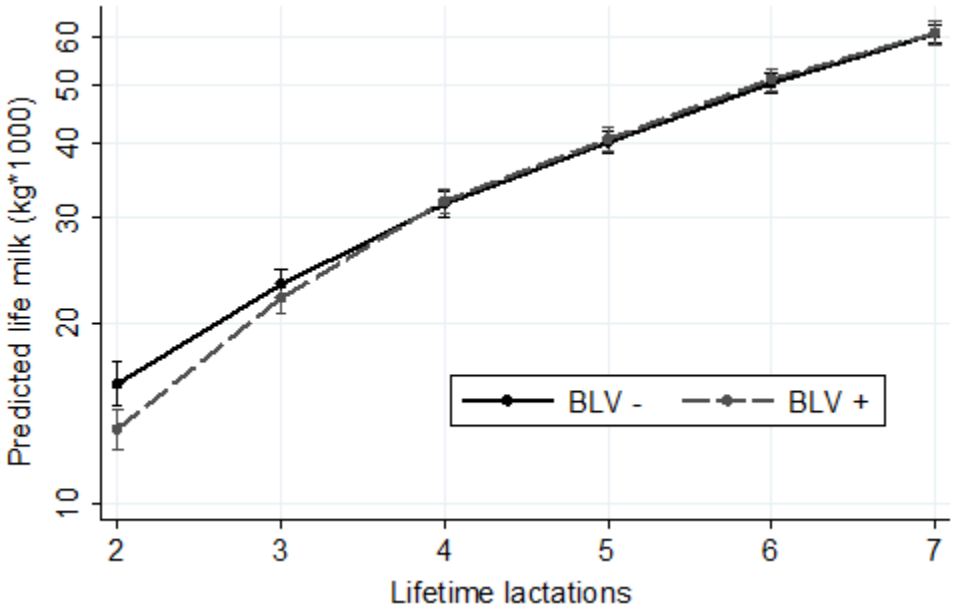

Figure 3.5. Interaction plot illustrating the relationship between predicted lifetime milk production (after back-transformation to original scale; Y-axis) and longevity (lifetime lactations; X-axis) by bovine leukemia virus (BLV) infection status, along with the corresponding 95% confidence intervals (spikes) for 4052 study cows.

CHAPTER 4

CARRYOVER OF BOVINE LEUKEMIA VIRUS ANTIBODIES IN SAMPLES FROM SHARED MILK METERS

This chapter has been published in the Journal of Dairy Science (without substantive change):

"Nekouei, O., J. Sanchez and G. Keefe. 2015. Carryover of bovine leukemia virus antibodies in samples from shared milk meters. J. Dairy Sci. 98: 5274-5279."

4.1 Abstract

Screening for infectious diseases of cattle using milk from the dairy herd improvement (DHI) sampling process is very convenient. However, when samples from shared milk meters are used, carryover of antibodies or other diagnostic targets can complicate the interpretation of the diagnostic test results for diseases, including bovine leukosis. The objectives of this chapter were: 1) to assess the potential for carryover of antibodies against bovine leukemia virus (BLV) in milk samples obtained from shared meters, and 2) to determine if adjustment of the diagnostic test cut-off value would improve the test characteristics for meter-collected milk ELISA results.

Eight dairy farms were randomly selected from herds with a wide range of BLV prevalence levels in Prince Edward Island (PE), Canada. Within each chosen farm, two to four milk meters were randomly selected. During the routine procedures of DHI sampling, two simultaneous milk samples, one hand-collected at the beginning of milking (after udder preparation), and the other from the corresponding milk meter were taken from all lactating cows (n = 236) that were milked at the selected meters (n = 26). The sequence of cows using each meter was recorded. All samples were tested for BLV antibodies using a commercial indirect ELISA. Antibody carryover potential was assessed in meter-collected samples, which were preceded by other cows using the same meters. Applying the hand-collected sample results as the reference standard, a new cut-off was defined for the meter-collected samples to optimize the test characteristics.

At the standard cut-off value of the diagnostic test, 110 (46.6%) of the hand-collected, and 136 (57.6%) of the meter-collected samples were positive. For low-titer cows (e.g. true negatives), the likelihood of antibody carryover significantly increased as

the titer of preceding cows increased, while this change was not substantial for high-titer cows. The odds of obtaining false diagnoses in meter-positive samples became larger with increase in the titer of preceding cows. A suspicious category for meter ELISA results was defined, and a retest was recommended for the cows falling into this category. This strategy effectively assisted in reducing the number of consequent false positive results. When DHI-collected samples are used, carryover can affect the interpretation of dichotomous test results, and may require adjustment of assay cut-off values.

4.2 Introduction

Enzootic bovine leukosis (EBL) is an economically important disease of dairy cattle caused by bovine leukemia virus (BLV). In North America, prevalence of the infection has been high and appears to have a rising trend (Samagh and Kellar, 1982; Richardson and Macaulay, 1992; Sargeant et al., 1997; VanLeeuwen et al., 2001; VanLeeuwen et al., 2005; VanLeeuwen et al., 2006; Bartlett et al., 2014). For instance, in Prince Edward Island, Canada, herd-level prevalence of BLV was 49.2% in 1989 (Richardson and Macaulay, 1992), increased to 63.3% in 1998 (VanLeeuwen et al., 2001), and is currently at 90% based on a survey of bulk tank milk in all dairy herds in the province completed in 2013 (Chapter 5). However, no broad-based national program for controlling EBL in Canada and the United States has been implemented.

A number of studies have recently been conducted to estimate the prevalence of BLV infection and define cost-effective screening tools to be applied in control programs. Monitoring meter-collected milk samples, obtained from the dairy herd improvement (DHI) process has become one of the standard and economically efficient procedures for screening for important infectious diseases in dairy cattle, such as bovine

viral diarrhea, Johne's disease, and EBL (Attalla et al., 2010; Sorge et al., 2011). Among the available commercial tests for detection of antibodies against BLV, milk ELISA is a desirable method in large-scale herd surveillance, because milk sampling during the DHI process is much more convenient and cost-effective than serum collection (Erskine et al., 2012).

With respect to sequential milk samples, carryover denotes the risk of inclusion of some residual milk from the previous cows in the subsequent milk samples (Ordolff, 1997; Lovendahl and Bjerring, 2006). With increasing utilization of DHI diagnostic services on meter-collected samples, there is a legitimate concern regarding carryover or cross-contamination of milk samples from shared milking equipment.

The objectives of this study were: 1) to assess the potential for carryover of BLV antibodies in milk samples obtained from shared meters, and 2) to determine if adjustment of the diagnostic test cut-off value would improve the test characteristics for meter-collected milk ELISA results.

4.3 Materials and methods

4.3.1 Sample collection

Based on a companion study using bulk tank milk samples from all dairy farms in Prince Edward Island (PE), Canada, all DHI-participant farms were assigned into 5 separate categories of BLV infection level: category 1) assumed uninfected, 2) low prevalence, 3) medium prevalence, 4) high prevalence, and 5) very high prevalence farms (Chapter 5). In July 2013, two farms were randomly selected from each of the categories 2 to 5 (8 farms in total). Within each selected farm, two to four milk meters were randomly selected, proportional to the lactating herd size (Table 4.1). During one round

of the routine DHI sampling procedure, two simultaneous milk samples (30 ml each), one hand-collected at the beginning of milking (after udder preparation), and the other from the corresponding milk meter, were taken from all lactating cows (n = 236) that were milked at the selected meters (n = 26). The sequence of the cows milked using each meter was precisely recorded by the project personnel.

4.3.2 Laboratory testing

Meter-collected samples were submitted to the Maritime Quality Milk (MQM) laboratory located at the Atlantic Veterinary College, University of Prince Edward Island, in Charlottetown, after undergoing the standard quality and components analyses in a local DHI laboratory (PEI Analytical Laboratory, Charlottetown, PE). Hand-collected samples were directly submitted to the MQM laboratory. All samples were tested for BLV antibodies using a commercial indirect ELISA kit (SVANOVIR BLV gp51-Ab, Svanova, Uppsala, Sweden). The test results were reported as percent positivity (PP) values [PP = ($OD_{corrected}$ sample/$OD_{corrected}$ positive control) × 100, where OD is optical density], and the recommended cut-off value of the kit for individual cow milk samples was 10.

4.3.3 Statistical analyses

All of the statistical analyses were conducted in Stata 13.1 (StataCorp, College Station, Texas. USA).

4.3.3.1 Agreement

To evaluate the overall agreement between hand (PP_{hand}) and meter (PP_{meter}) test results, a scatter diagram was produced and the concordance correlation coefficient was calculated. In addition, the overall agreement of the dichotomized results (at the

recommended cut-off of 10) from the two types of samples was explored using McNemar's Chi-square test and Cohen's kappa coefficient (Dohoo et al., 2009).

4.3.3.2 Carryover effects

In order to determine the potential carryover effects of BLV antibodies, all cows which had been preceded by other cows at the same meters, contributed to building a multivariable linear regression model. It was assumed that the difference between meter- and hand-collected ELISA values for each cow ($Y = PP_{meter} - PP_{hand}$) was a function of hand value for the cow (PP_{hand}; X_1), meter value from the preceding cow ($PP_{meter-1}$; X_2), and their interaction term (X_1X_2). The final model was as follows:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_1X_2 + \varepsilon$$

In the equation, β_0 is the intercept; ε is the residuals of the model; and other βs are regression coefficients corresponding to the terms defined above; Y represents the potential carryover effects. To control for the potential clustering effects of herds, robust standard errors were applied to the estimates.

A one-sample T-test was performed to determine whether the average of the difference (Y) values for the cows, which were milked first per meter (first-per-meter cows) was significantly different from zero.

4.3.3.3 Carryover in meter-positive samples

To specifically explore the impact of carryover on generating extra false positive results to BLV infection, all meter-positive cows ($PP_{meter} \geq 10$) that were preceded by other cows at the same meters were extracted (n = 133; three missing values). The difference between the hand value of each cow (PP_{hand}) and the hand value from the previous cow (PP_{hand-1}) using the same meter was then calculated ($PP_{hand} - PP_{hand-1}$). A

logistic regression model was built to determine the impact of this difference, as a surrogate for potential carryover, on the odds of obtaining a false positive diagnosis (positive in meter, but negative in hand samples), as compared to the odds of a true positive diagnosis (positive in both types of the samples).

4.3.3.4 ELISA cut-points

Finally, to explore new ELISA cut-off values that would minimize the proportion of false positives in the routine application of the test on meter samples, a two-graph receiver operating characteristic (ROC) analysis was carried out (Dohoo et al., 2009). The optimal adjusted cut-off for meter-collected samples was determined using the dichotomized hand-collected results as the reference standard. Based on the original kit and the adjusted cut-off values for the meter-collected samples, three categories of ELISA results (negative, suspicious, and positive) were defined.

4.4 Results

Descriptive data on study herds and samples are presented in Table 4.1. Lactating herd size in the study herds ranged from 46 to 126. Between 12 and 48 cows from the study herds were sampled for a total of 236 cows (all Holstein). Based on the recommended cut-off of the test, 110 cows (46.6%) in the hand-collected samples, and 136 cows (57.6%) in the meter-collected samples were found to be positive.

4.4.1 Agreement

The McNemar’s Chi-square test was highly significant ($P < 0.001$), suggesting that the proportion of positive test results disagreed between the dichotomized hand- and meter-collected results. Because the McNemar's test was significant, the Cohen's kappa

result is not presented. When examined on a continuous basis, there was a strong correlation [concordance correlation coefficient = 0.905 (95% CI: 0.882-0.928)] between the two sets of the PP values. This relationship is illustrated in Figure 4.1.

4.4.2 Carryover effects

The final linear model (Table 4.2) included 207 cows (25 first-per-meter cows and one missing; as well as 3 cows with unavailable previous values). The interaction term between PP_{hand} and $PP_{meter-1}$ was highly significant ($P < 0.001$), indicating that the behavior of antibody carryover against the preceding cows' meter titers depended on different levels of cows' hand titers. To clarify this interaction, a graph was produced to illustrate changes in Y for the cows versus titer of the preceding cows ($PP_{meter-1}$) at three different constant levels of PP_{hand} values (Figure 4.2). From the graph, for the cows with low levels of BLV antibody titer (e.g. $PP_{hand} < 30$; including all negatives), predicted antibody carryover (Y) increased as the titer of their preceding cows increased. As the titer of cows increased (e.g. $PP_{hand} > 60$), antibody carryover started showing a weaker positive association with changes in the titer of previous cows, until it gradually faded towards being insignificant. The T-test for the first-per-meter cows resulted in a P-value of 0.289, indicating the average of Y values was not significantly different from zero. In other words, when there was no possibility for carryover at all, the average of measurement differences between hand- and meter-collected values would be minor.

4.4.3 Carryover in meter-positive samples

Based on the simple logistic model, the carryover effect in positive samples was highly significant ($P < 0.001$). With each unit (percent positivity) increase in $PP_{hand} - PP_{hand-1}$, the log-odds of getting a false diagnosis decreased by 0.050 (95% CI: 0.031-

0.067); i.e. preceding by a high titer cow at the same meter, significantly increased the odds of false positive results in the subsequent meter-positive samples.

4.4.4 ELISA cut-points

Figure 4.3 illustrates the two-graph ROC curve (sensitivity/specificity versus all possible cut-off values). According to the graph, the cut-point for optimizing test characteristics would be achieved at a PP of approximately 45. If the later cut-off was used alone, we would gain a quite higher specificity (from 0.777 to 0.984), while the loss in sensitivity would be relatively negligible (from 0.982 to 0.955). Consequently, the number of false positive results substantially reduced (from 28/136 to 2/106), whereas the impact on the false negatives was relatively small (increased from 2/100 to 6/130). As presented in Table 4.3, three categories of the ELISA values were defined as: 1) negative ($PP_{meter} < 10$); 2) doubtful or suspicious ($10 \leq PP_{meter} \leq 45$); and 3) positive ($PP_{meter} > 45$). Thirty samples were located within the suspicious range, and 86.7% (26/30) of those were falsely positive (i.e. negative in hand-collected samples), whereas only 1.9% (2/106) of the samples within the positive range were falsely positive.

4.5 Discussion

In our study, we found a significant carryover when cows with high BLV antibody titers preceded low-titer (e.g. true negative) cows at the same meters. For instance, based on the interaction plot, if a negative cow was preceded by a positive cow with medium-to-high titer (e.g. > 50), adequate levels of carried BLV antibodies could be detected and led to a false positive diagnosis. In high prevalence herds, where there is a higher chance of meter contamination, and therefore risk of carryover, substantial number of non-infected cows may be falsely diagnosed at the published cut-off. Different studies

have evaluated the importance of carryover of milk components (e.g. fat, protein, and somatic cell count) in samples from automated milking systems (Dill, 1974; Ordolff, 1997; Friggens and Rasmussen, 2002; Lovendahl and Bjerring, 2006). For conventional milking meters, Dill (1974) indicated a significant carryover effect on the milk fat when the previous sample was as much as 1% higher or 2% lower in fat than the tested sample. Byrem et al (2013) stated that when measuring endogenous milk components such as fat and protein, the influence of existing carryover was negligible, but this effect could particularly be important on exogenous components (such as antibodies or other diagnostic targets). They further suggested that carryover of 3% milk between samples could result in detectable levels of antibody in milk in an uninfected cow if preceded by an infected cow having milk antibody levels at relatively low levels (Byrem et al., 2013). Despite this, we were not able to find any published data on antibody carryover in conventional milk meters.

To account for BLV antibody carryover, we defined three levels of sample results for optimizing the performance of the current ELISA test. Samples with PP_{meter} <10 were negative and those > 45 were positive, while samples with $10 \leq PP_{meter} \leq 45$ were considered a suspicious category. As indicated in Table 4.3, the majority of cows in the suspicious range were in fact negative. Hence, if a cow falls within this range, a retest should be requested. The retest sample should be a direct hand-collected milk sample or a serum sample. Subsequently, the second test result should be interpreted based on the original dichotomous cut-off of the kit. Putting this strategy in place, a substantial number of false positive diagnoses could efficiently be prevented without loss in sensitivity (i.e. no extra false negatives are generated). This gain can economically be

significant when test and removal, or test and segregation programs for BLV infection are pursued. For herds with medium or high levels of within-herd prevalence, such as many dairy farms in North America, management strategies for controlling BLV are preferred due to considerable cost (and/or impracticality) of removal and segregation programs (Sandev et al., 2000; Bartlett et al., 2014). Therefore, our recommendations could provide a more cost-effective screening or monitoring tool for accomplishing the control measures.

Other methods for controlling the carryover effects have also been proposed, such as diluting meter milk samples before testing (Walsh et al., 2013). This method could potentially cause extra false negative results for low-titer positive samples, and interfere with precise measurements on milk components during DHI procedures. A potentially useful method for avoiding carryover effects could be recommending rinsing of milk meters between cows. This procedure is likely to reduce carryover, but may be difficult to consistently adhere to because of time pressures during milking on many dairy farms. Despite the challenges identified in this research, DHI-collected samples offer many economical and logistic advantages for screening for BLV (or other common pathogens in dairy cattle).

Overall, we had only two (0.8%) false negative results (i.e. negative in meter, but positive in hand-collected samples). These were identified as outliers during the statistical analyses; i.e. the two cows with a large difference between their hand and meter ELISA values ($Y > 40$). However, these two cows were kept in our analyses, because 1) refitting the linear model without the two outliers did not affect the conclusions, and 2) we could not find any logical reason (e.g. obvious laboratory mistakes) for removing them. One of

the false negative samples was from a first-per-meter cow (i.e. no possibility for carryover), while the other one was preceded by a positive cow. Moreover, the linear model suggested that the potential carryover effect was not significant when low-titer (or even medium-titer) positive cows were preceded by low-titer cows at the same meters. According to our results, therefore, the potential diluting effects of carryover on generating extra false negative results would not be concerning. Hence, we did not consider changing the interpretation of the lower ELISA cut-off of 10.

This study was only focused on carryover of BLV antibodies using an ELISA test. However, the issue of carryover might be of concern in diagnosing other important infectious diseases of dairy cattle through DHI-process, particularly when methods with high analytical sensitivity such as ELISA and PCR are applied. Therefore, conducting similar studies on the other commonly tested pathogens is recommended to clarify and mitigate such detrimental effects.

4.5.1 Conclusions

Carryover of BLV antibodies at shared milk meters was significant. For low-titer cows (e.g. true negatives), the carryover effect was positively associated with the titer of the preceding cows. This could result in generating false positive results in the BLV antibody-ELISA test on meter-collected samples from DHI procedures. For meter-collected samples, if we only rely on the dichotomous test results with the original cut-off, the consequences can be economically substantial. Thus, defining a suspicious category for the ELISA titers, and recommending a retest on the samples falling within this range would be very helpful in reducing the false positive rate.

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Table 4.1. Herd characteristics and Milk-ELISA results for antibodies against bovine leukemia virus (BLV) on 8 study farms from Prince Edward Island, Canada

Farm	Prevalence level[1]	Lactating herd size	Selected meters	Tested cows	Positives in meter[2]	Positives in hand[2]
1	Low	49	3	27	0	0
2	Low	83	4	28	4	4
3	Medium	57	4	22	15	11
4	Medium	46	2	12	5	4
5	High	62	3	30	24	23
6	High	126	4	48	35	25
7	Very high	76	3	37	27	23
8	Very high	64	3	32	24	20
Total	-	563	26	236	136	110

[1]Predicted levels of within-herd prevalence of BLV, based on a companion study (Chapter 5).
[2]Number of cows testing positive at the recommended ELISA cut-off of 10 (percent positivity).

Table 4.2. Results of the final linear regression model, evaluating the effect of ELISA titers (X) for 207 study cows on the potential carryover (Y) of antibodies against bovine leukemia virus.

Variable [a]	Coefficient	SE	95% CI	
X1	0.009	0.064	-0.143	0.167
X2	0.186	0.028	0.119	0.253
X1X2	-0.003	0.001	-0.004	-0.002
Constant	3.501	1.906	-1.006	8.007

[a] X1: hand ELISA value for a cow; X2: meter ELISA value for the preceding cow (before X1); X1X2: the interaction term between X1 and X2.

Table 4.3. Cross-classification of milk ELISA results for antibodies against bovine leukemia virus (BLV) from paired hand- and meter-collected samples in 236 study cows.

	Meter samples[1]			
Hand samples	$PP_{meter} < 10$	$10 \leq PP_{meter} \leq 45$	$PP_{meter} > 45$	Total
	-	Suspicious	+	
-	98	26	2	126
+	2	4	104	110
Total	100	30	106	236

[1]The range of ELISA results in percent positivity for meter-collected samples (PP_{meter}) has been divided into 3 categories at the two cut-off values of 10 (original) and 45 (adjusted).

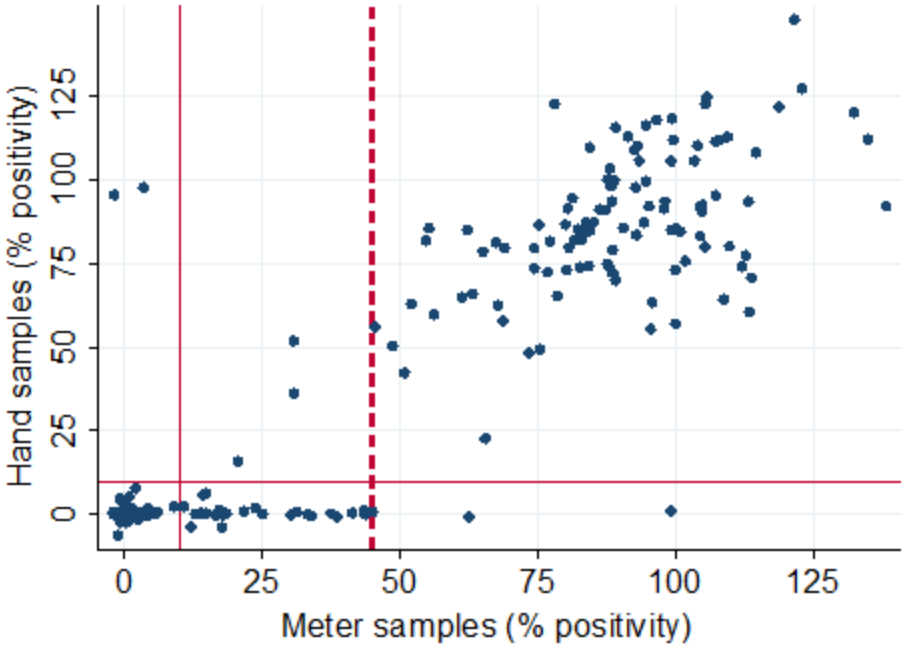

Figure 4.1. Scatter plot of Milk-ELISA results (percent positivity) for antibodies against bovine leukemia virus in paired hand- and meter-collected milk samples from 236 study cows. The two overlaid solid lines represent the recommended cut-off of 10, and the dashed line represents the adjusted cut-off of 45.

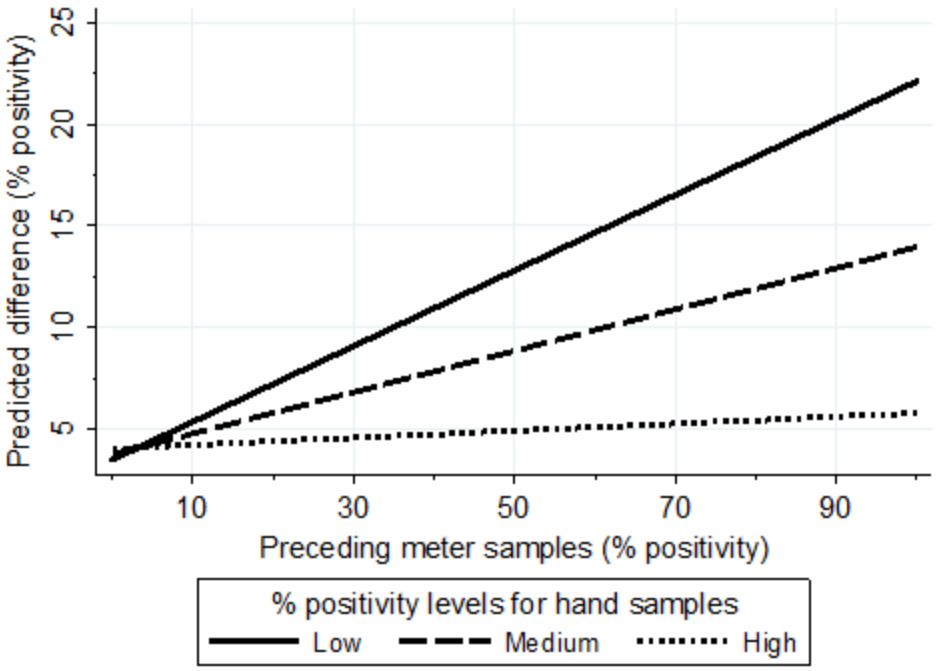

Figure 4.2. Interaction plot illustrating the realtionship between predicted difference of paired hand- and meter-collected ELISA results (Y-axis; PPmeter - PPhand) and meter-collected results of preceding cows (X-axis; PPmeter-1), at different levels of hand-collected results (PPhand) for antibodies against bovine leukemia virus in 207 study cows. Low, medium, and high levels of antibody represent 0, 30, and 60 percent positivity values for hand-collected samples, respectively.

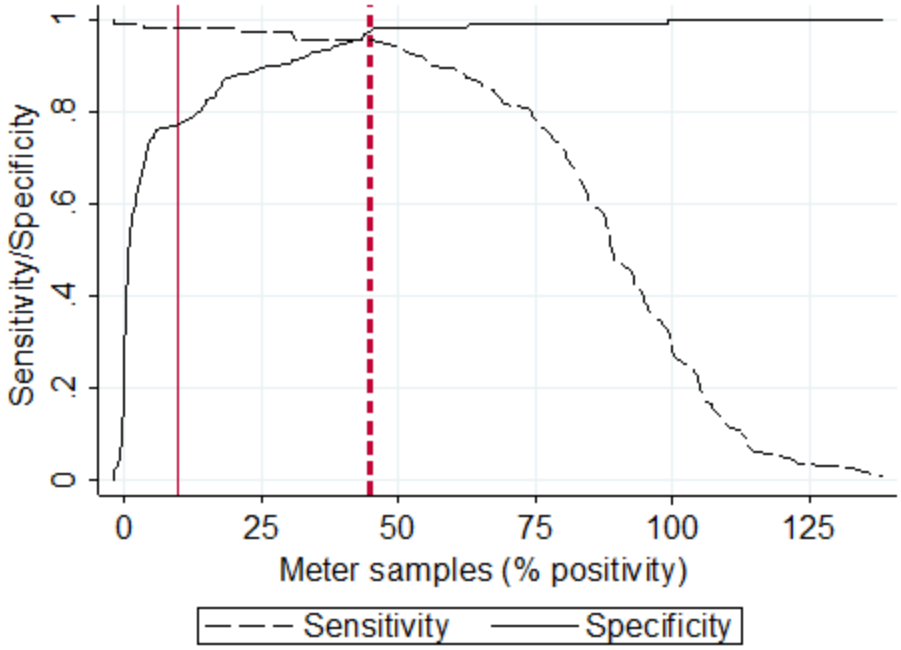

Figure 4.3. Sensitivity/specificity plot against all possible cut-off values for paired hand- and meter-collected Milk-ELISA results for bovine leukemia virus antibodies in 236 study cows. Hand samples were the dichotomized reference standard to the continuous meter values (percent positivity). The vertical solid line represents the recommended cut-off of 10, and the vertical dashed line represents the adjusted cut-off of 45.

CHAPTER 5

PREDICTING WITHIN-HERD PREVALENCE OF INFECTION WITH BOVINE LEUKEMIA VIRUS USING BULK-TANK MILK ANTIBODY LEVELS

This chapter is in press in the Preventive Veterinary Medicine (without any change):

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5.1 Abstract

Enzootic bovine leukosis (EBL) is an economically important infection of dairy cattle caused by bovine leukemia virus (BLV). Estimating the prevalence of BLV within dairy herds is a fundamental step towards pursuing efficient control programs. The objectives of this study were: 1) to determine the prevalence of BLV infection at the herd level using a bulk-tank milk (BTM) antibody ELISA in the Maritime region of Canada (3 provinces); and 2) to develop appropriate statistical models for predicting within-herd prevalence of BLV infection using BTM antibody ELISA titers.

During 2013, three monthly BTM samples were collected from all dairy farms in the Maritime region of Canada (n = 623) and tested for BLV milk antibodies using a commercial indirect ELISA. Based on the mean of the 3 BTM titers, 15 strata of herds (5 per province) were defined. From each stratum, 6 herds were randomly selected for a total of 90 farms. Within every selected herd, an additional BTM sample was taken (round 4), approximately 2 months after the third round. On the same day of BTM sampling, all cows that contributed milk to the fourth BTM sample were individually tested for BLV milk antibodies (n = 6111) to estimate the true within-herd prevalence for the 90 herds. The association between true within-herd prevalence of BLV and means of various combinations of the BTM titers was assessed using linear regression models, adjusting for the stratified random sampling design.

Herd-level prevalence of BLV in the region was 90.8%. In the individual testing, 30.4% of cows were positive. True within-herd prevalences ranged from 0 to 94%. All linear regression models were able to predict the true within-herd prevalence of BLV reasonably well ($R^2 > 0.69$). Predictions from the models were particularly accurate for

low-to-medium spectrums of the BTM titers. In general, as a greater number of the four repeated BTM titers were incorporated in the models, narrower confidence intervals around the prediction lines were achieved. The model including all 4 BTM tests as the predictor had the best fit, although the models using 2 and 3 BTM tests provided similar results to 4 repeated tests. Therefore, testing two or three BTM samples with approximately two-month intervals would provide relatively precise estimates for the potential number of infected cows in a herd. The developed models in this study could be applied to control and eradication programs for BLV as cost-effective tools.

5.2 Introduction

Enzootic bovine leukosis (EBL) is an important infection of dairy cattle worldwide which is caused by bovine leukemia virus (BLV). The virus is transmitted through infected blood lymphocytes (Gillet et al., 2013). Premature culling, death, and condemnation of carcasses at slaughter due to lymphoma, impaired immune function, as well as restrictions on international trade of infected cattle and their products are among the most significant economic losses attributed to the disease (Sandev et al., 2000; Bartlett et al., 2014).

Many European countries, including the UK, France, Germany, Spain, the Scandinavian countries, Belgium, and the Netherlands, are officially free from EBL (Annual EU report, 2013). Some other countries, such as Japan, the United States, and Argentina, have actively been working on addressing their BLV problems in recent years in order to develop cost-effective programs for their dairy industries (Ott et al., 2003; Monti et al., 2007; Murakami, 2009; Rodriguez et al., 2011).

In different provinces of Canada, there have been a number of serological studies which have estimated prevalence and impact of BLV infection. In 1980, the national prevalence of BLV infection in Canadian dairy herds was estimated at 40.5%, while only 9.3% of tested cattle were positive (Samagh and Kellar, 1982). However, 15 to 20 years later, infection levels appeared to have substantially increased. Sargeant et al. (1997) indicated 69.6% of the 102 tested dairy herds, and 23% of the 1330 tested cows in Ontario were positive to BLV. VanLeeuwen et al. (2001) reported that 70% of herds in the Maritime region of Canada (including provinces of Prince Edward Island (PE), New Brunswick (NB), and Nova Scotia (NS)) had at least one infected cow, while the prevalence of infection at the cow level was estimated at 20.8%. Similar studies have revealed a high prevalence of BLV infection across the country (VanLeeuwen et al., 2005; VanLeeuwen et al., 2006; Scott et al., 2006). Nevertheless, there is still no broad-based program for controlling EBL in Canada.

Control of EBL at the national level usually consists of one or more of the following approaches: management interventions; test and segregation; and test and slaughter. Selection and success of each strategy are heavily dependent on having a reliable estimate of the within-herd prevalence (Bartlett et al., 2014). Attaining a reasonably valid estimate of the within-herd prevalence would be a fundamental step towards pursuing efficient control and eradication programs for BLV in every dairy herd. Individual serum or milk sampling from all cows on a dairy farm would provide an accurate measure of BLV infection prevalence; however, it would demand a great deal of time, labour, and cost. Therefore, in order to motivate farmers and veterinarians to

maximum participation in future comprehensive control programs, a cost-effective screening or monitoring tool for BLV at the herd level would be desirable.

Using bulk-tank milk (BTM) samples, collected by the dairy herd improvement (DHI) companies, has become one of the most convenient and economically efficient mechanisms for screening for important infectious diseases in dairy cattle (Houe et al., 1995; Attalla et al., 2010; Sorge et al., 2011). For instance, BTM ELISA has frequently been applied to surveillance of EBL, Johne's disease, and bovine viral diarrhea (Niskanen, 1993; Bitsch and Ronsholt, 1995; Reber et al., 2012; Nielsen and Toft, 2014). Once cattle become infected with BLV, they remain infected for life and generate a continuous antibody response. This characteristic adds to the credibility of antibody-based diagnostic techniques for BLV (Radostits et al., 2006; Monti et al., 2007). Among the available commercial tests for detection of antibodies against BLV, milk ELISA is a desirable method in large-scale herd surveillance, which has often been used for classification of herds as infected or non-infected (Erskine et al., 2012). However, there has been no evaluation of the predictive ability of BTM ELISA tests for within-herd prevalence of BLV.

The objectives of this study were: 1) to determine the prevalence of BLV infection at the herd level using a BTM antibody ELISA in the Maritime region of Canada ; and 2) to develop applied statistical models for predicting within-herd prevalence of BLV infection using BTM antibody ELISA titers.

5.3 Materials and methods

5.3.1 Herd selection for determining herd-level prevalence, and sample collection (a census)

All dairy farms in the Maritime region of Canada (n = 644, in 2013) were the target and source populations for the herd-level prevalence part of the study. Permission was granted from the governing bodies of dairy producers of the three Maritime provinces to obtain BTM samples collected by these bodies for regulatory purposes in order to conduct our study. Therefore, all dairy farms in the Maritime region were the study population.

During 2013, three bulk-tank milk samples (30 ml each), taken at one-month intervals, were obtained by the milk truck drivers on their routine milk pick-ups from the Maritime dairy farms. The drivers followed the standard procedures used for collection of samples for regulatory and payment purposes and, therefore, samples were well mixed.

The milk samples were kept at 4^{o}C until BLV laboratory testing commenced. All samples were tested for BLV antibodies using an indirect ELISA test (see subsection 2.3). The test results were reported as percent positivity (**PP**), and the cut-off value for a positive result on the test kit for pooled (bulk tank) milk samples was $\geq$ 5, according to the manufacturer's specifications.

5.3.2 Herd and animal selection for predicting within-herd prevalence, and sample collection

From the study herds in the census, a subset of herds (n = 90) was selected to evaluate the association between the BTM titers and within-herd prevalence of BLV infection. Two steps were taken to create pools of eligible farms with a broad spectrum of within-herd prevalences for the stratified random sampling strategy used in this part of

the study: 1) creation of five pools (strata) per province of increasing BTM-based herd-level prevalence; and 2) identification of farms subscribing to monthly DHI testing.

For step 1, the arithmetic mean of the three monthly ELISA titers for each farm was calculated. Based on the test cut-off, and the distribution of the test means (Figure1), all study farms were assigned into one of five strata within each of the three study provinces (a total of 15 strata), as follows:

1) Potentially uninfected or very low-prevalence farms (mean < 5)
2) Expected low-prevalence farms (5 ≤ mean < 40)
3) Expected medium-prevalence farms (40 ≤ mean < 55)
4) Expected high-prevalence farms (55 ≤ mean < 70)
5) Expected very high-prevalence farms (70 ≤ mean)

The reason for this stratification was to ensure that we would get BTM percent positivity titers across the range of possible ELISA test values (e.g. 0 to 100). This will lead to the most precise and representative estimates for the coefficients in our final regression models.

For step 2, the source population was restricted to all DHI-registered dairy farms in the region (67% of all dairy farms), because of the necessary access to their monthly individual cow milk samples and information. Based on the number of DHI farms in each province, available budget, and other logistical considerations, 30 dairy farms from each province were targeted for recruitment. Using computer generated random numbers, six farms from each of the 15 strata were randomly selected for a total of 90 farms. An informed consent was obtained from each selected farm for conducting the BLV tests on their individual cow milk samples and further access to the farm information. All

procedures were approved by the University of Prince Edward Island Animal Care Committee.

Within the 90 selected farms, an additional BTM sample (fourth round) was taken, approximately 2 months after the third round. On the same day, all lactating cows that contributed milk to the fourth BTM samples (n = 6111) were individually sampled via the corresponding milk meters used in every selected herd. From each milk meter, a 30 ml milk sample was taken by the person who routinely took the DHI samples for Valacta (Sainte-Anne-de-Bellevue, QC) and one BROTAB (Sierra Court, CA, USA) was added to help preserve the sample for testing. Samples were kept at 4^{o}C until all testing was completed. Based on the individual cow milk ELISA cut-off of 45 (Chapter 4), the proportion of positive milking cows to BLV antibodies (PP > 45) in each herd was determined.

5.3.3 Laboratory testing

After undergoing the standard components and quality analyses in the local laboratories in each province, the BTM samples were preserved with one BROTAB (Sierra Court, CA, USA) and transferred to the Maritime Quality Milk (MQM) laboratory located at the Atlantic Veterinary College, University of Prince Edward Island, in Charlottetown to be tested for BLV antibodies.

All of the collected individual cow milk samples were submitted to the DHI laboratory in Charlottetown (PEI Analytical Laboratory, Charlottetown, PE) for routine DHI testing. Subsequent to the standard components and quality analyses, the samples were transferred to the MQM laboratory for BLV testing.

All BTM and individual cow milk samples were tested within a maximum of seven days from the sampling dates using a commercial indirect ELISA kit (SVANOVIR BLV gp51-Ab, Svanova, Uppsala, Sweden). The test results were reported as percent positivity values [PP = ($OD_{corrected}$ sample/$OD_{corrected}$ positive control) × 100, where OD is optical density].

5.3.4 Statistical analyses

All of the statistical analyses were carried out in Stata 13.1 (StataCorp, College Station, TX. USA).

5.3.4.1 Descriptive statistics

If a BTM test was positive (PP $\geq$ 5) in any of the three rounds of sampling, the farm was considered infected with BLV for the herd-level prevalence calculations.

True within-herd prevalence of BLV infection (TP) was estimated for each of the selected herds using the following formula (Dohoo et al., 2009):

$$TP = (AP + Sp - 1)/(Se + Sp - 1)$$

Where AP is apparent prevalence of BLV infection within the study herds; sensitivity (Se) and specificity (Sp) of the individual ELISA test at the applied threshold of 45 were 0.955 and 0.984, respectively (Chapter 4).

Correlations between the repeated BTM ELISA results for all herds were assessed by calculating the concordance correlation coefficients between every pair of the sampling rounds.

5.3.4.2 Analytical statistics

Several linear regression models were built to predict the true within-herd prevalence for the 90 selected herds based on their BTM ELISA titers. To satisfy the

assumptions of the linear regression models, the estimated true within-herd prevalence of BLV was square-root transformed and used as the outcome of interest (Y), and the means of plausible combinations of the BTM ELISA titers were examined to serve as the predictor of interest (X), in six separate modeling scenarios as follows:

1) Only round 4 (concurrent BTM and individual cow milk samples; X_1)
2) Mean of rounds 4 and 3 (X_2)
3) Mean of rounds 4, 3, and 2 (X_3)
4) Mean of rounds 4, 3, and 1 (X_4)
5) Mean of all 4 rounds (X_5)
6) Mean of rounds 3, 2, and 1 (X_6)

Number of lactating cows within each herd (lactating herd size) was included in all models to control for the potential dilution effects due to pooled milk in the bulk tanks. The stratified random sampling design was also taken into account in all of the scenarios, by incorporating sampling weights and finite population corrections corresponding to each of the 15 strata. A diagram for predicted within-herd prevalence versus BTM ELISA titers was generated to illustrate and compare the resultant functions from the six final models.

The within-herd prevalence of BLV for all study farms was predicted and graphed in order to exhibit a general picture of the infection levels in the regional dairy herds. For this purpose, the means of the first three monthly BTM ELISA titers for each herd were used as the predictor (corresponding to scenario X_6).

5.4 Results

Overall, 623 dairy farms (97% of all regional dairy farms in 2013) completed the first part of the study. Of the 623 herds, 566 (90.8%) were found to be positive to BLV antibodies on at least one of the three monthly BTM ELISA tests. Based on these results, herd-level prevalence of BLV in the Maritimes is presented in Table 1. As depicted, the three study provinces had quite similar herd-level prevalences and there was no apparent difference in the infection level between DHI-registered herds and all herds collectively.

Concordance correlations between the three monthly BTM ELISA titers for all herds, as well as between the four rounds for the 90 selected herds are presented in Table 2. The calculated coefficients indicated reasonably high correlations (minimum of 72.3%) between the results from all pairs of the sampling rounds.

The mean lactating herd size for the 90 selected herds for individual testing was 68 (range: 23 – 287), and 78 herds (87%) had fewer than 100 lactating cows. In the individual testing, 30.4% (1860/6111) of the cows were positive. Among the tested cows, 90% were Holstein. True within-herd prevalence of BLV infection ranged from 0 to 94%.

Lactating herd size was found to be clearly non-significant in all of the examined scenarios ($P > 0.2$) and it did not change the other estimates in the linear regression models (of the square root of true within-herd prevalence of BLV). As a result, it was not included in the final models. Final models for the six scenarios of interest are provided in Table 3. A strong positive association ($P < 0.001$) between the square root transformed true within-herd prevalence and BTM ELISA titer was identified in all scenarios. When

only concurrent BTM results were used as the predictor (X_1), two significant outliers were detected. Incorporating BTM results from the preceding rounds improved the fit and predictive ability of the models (increasing the corresponding coefficient of determination, R^2).

Figure 2 illustrates the final prediction functions, by the various scenarios. According to the graph, there is no clear distinction in the predicted within-herd prevalence among the functions for low-to-medium values of BTM ELISA (i.e. PP < 55). For instance, a herd with PP = 40 would roughly be expected to harbor 17.8 % (16.0-19.7%) and 18.1% (12.9-25.3%) antibody-positive lactating cows, using the two extreme scenarios, X_5 and X_1 respectively. Incorporating more rounds of BTMs led to a narrower uncertainty (greater precision) about the prediction lines, especially at low percent positivity (Appendix B). As the BTM titer increased, above 55, the corresponding predicted within-herd prevalences became more divergent among the models; but this variation still did not appear to be substantial.

A scatter plot displaying the association between the predicted within-herd prevalence and the mean value of all repeated BTM ELISA titers (scenario X_5) for the selected 90 herds is presented in Figure 3, along with the line of best fit and its 95% confidence interval. The curve seems to fit the data well, and the confidence interval around the prediction line is fairly narrow, expanding somewhat as the prevalence increases.

Using the X_6 model, predicted within-herd prevalences for all herds in the region (n = 623) were determined from the means of the first three monthly BTM ELISA titers to exhibit a general picture of the infection levels in the region. Results suggested that

41.4% of the herds (n = 258) had potentially > 50% BLV-positive lactating cows, with 39 herds (6.9% of the total positive herds) being > 90% BLV-positive. Mean of the predicted within-herd prevalences for infected herds (n = 566) was estimated at 0.487 (SD = 0.255). Figure4 illustrates the frequency distribution of these predicted within-herd prevalences of BLV infection for the infected herds.

5.5 Discussion

Our results indicated that the prevalence of infection with BLV at the herd level in the Maritime region of Canada was very high. This is most likely due to dairy farmers in Canada not effectively controlling BLV transmission (Chapter 2). Herd-level prevalence of BLV infection in the Maritime region rose from 70% (60.3% - 79.7%) in 1998 (VanLeeuwen, et al., 2001) to over 90% in 2013 (present study). In the United States, as a part of the 2007 national dairy study (APHIS-USDA report, 2008), 83.9% of tested herds were found to be positive using BTM ELISA. Volunteer programs for certifying BLV-free status have been implemented by a small percentage of the dairy herds in North America (e.g. genetically valuable herds), in order to authorize the exportation of their genetic products to other countries, which demand a BLV-negative status (Reed, 1981; Bartlett et al, 2014). All of the available evidence indicates the necessity for decisive action against EBL. However, this is not appealing to the industry unless it employs inexpensive, efficient tools such as the suggested modeling approach in this study.

Measuring antibodies and other diagnostic targets in BTM as an indicator of herd status has historically been used for monitoring and control of important infectious diseases in dairy cattle, including EBL, BVD, and Johne's disease (Eloit et al., 1990; Beaudeau et al., 2001; Attalla et al., 2010; Sorge et al., 2011). Our results demonstrate

that BTM ELISA is not only a highly cost-effective tool to dairy herds for monitoring and screening for BLV infection, but also for predicting the within herd prevalence. Application of BTM antibody ELISA results for predicting the potential number of infected cows within herds would lay a strong foundation for pursuing economical BLV control programs.

In order to develop a reliable, applied statistical model for prediction of the within-herd prevalence, we were required to achieve reasonably strong correlations: 1) between the repeated rounds of BTM ELISA measurements, confirming the consistency of the test results between samples; and 2) between the outcome variable (true within-herd prevalence) and the BTM ELISA titer (i.e. attaining an acceptable R^2 in the final models). In our study, correlations between the repeated rounds of BTM titers were over 0.72 in all pairings. The correlation was above 80% for contiguous monthly BTM samples, but slightly decreased with longer than monthly time intervals between the samples. In general, the prevalence of BLV in herds from endemic areas remains relatively steady over time (Radostits et al., 2006). This characteristic of BLV infection would add to the credibility of prediction methods. Fluctuations in the repeated BTM titers could be mainly attributable to changing composition of lactating herds at sampling (i.e. every month, a number of milking cows are dried off, and some fresh heifers or dry cows join the milking herd after parturition). Addition or elimination of certain cows can influence the BTM titer. Cows in advanced stages of the infection (e.g. persistent lymphocytosis) often produce high levels of virus and circulating antibodies (Juliarena et al., 2007). Another source of variation between repeated BTM titers could be differences in laboratory measurements over time (Nielsen, 2002). As for the second requirement, the

R^2 of the models containing at least 2 BTM samples all were above 75% (Table 3), demonstrating good predictive ability to the proposed BTM methodology for predicting true within-herd prevalence.

Although using the concurrent BTM sample as the only predictor (X_1) led to acceptable estimates for BLV within-herd prevalence, the uncertainty around the prediction line was considerably wider than for the other scenarios using at least two BTM samples, particularly at low prevalences (Appendix B). Because this scenario also had poorer fit compared to the other scenarios, we do not recommend application of the one-sample strategy in practice. In contrast, incorporating more of the BTM samples collected over appropriate time intervals, and using the corresponding means as the model predictor, yielded more reliable results. As additional BTM rounds were engaged in the models, fit and predictive ability of the models improved, and outliers were not as extreme as they were in scenario X_1 (Appendix B). However, using all 4 rounds of BTM results (X_5) did not add substantial gain compared to the other scenarios (X_2-X_4 and X_6), and demanded more cost and effort. Recommendations should be made in light of trade-offs between a farmer's desire for additional precision (dependent on the management goals) versus the delays and costs imposed from additional rounds of BTM sampling. For instance, if only a basic sense of within-herd prevalence of infection with BLV was needed, model X_2 (2 samples, 2 months apart) could be adequate. In contrast, a slightly more accurate estimate of the within-herd prevalence can be generated using 3-sample models such as X_4. In high-prevalence herds (PP > 55), in order to monitor and document a decreasing trend of the prevalence due to an undertaken control program, models which provide greater accuracy would be recommended. Farmers with strong interest in

controlling BLV could adopt ongoing BTM screening. The two-month intervals would likely take most dry cows from the preceding rounds of sampling into account.

Despite the fact that our source population in this study was DHI-registered dairy farms (410/623), the source population was representative of our target population with respect to structure, management, and production parameters. In addition, there was no substantive difference in the herd-level prevalence of BLV between DHI herds and all herds (as presented in Table 1). Therefore, the corresponding model using the first three tests (X_6) was applied to predict the within-herd prevalence for all regional dairy farms. As our results suggested, within-herd prevalence of BLV in infected herds in the region was also high, which further emphasized the importance of taking immediate control measures.

The persistent nature of BLV infection, continuous antibody response, and absence of a vaccination make this pathogen a good candidate for integrating exact, quantitative prediction models into control programs, unlike other important infections of dairy cattle (e.g. BVD and Johne's disease) (Radostits et al., 2006; Gutierrez et al., 2014). Nielsen and Toft (2014) demonstrated a significant association between repeated BTM ELISA results and within-herd prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Danish dairy herds. However, the practical application of BTM testing for MAP was found to be limited because the BTM responses were in a relatively narrow range of the ELISA test, as well as due to substantial uncertainty around the prediction line. In the present research, we initially designed our study (implementing a stratified random sampling strategy) to obtain BTM titers over an expected full spectrum of ELISA values. Consequently, relatively narrow confidence

intervals about the prediction lines were achieved. Eiras et al. (2012) reported strong correlations between the within-herd seroprevalence and BTM antibody levels against BVD virus, using different ELISA methods in Galicia, Spain; however, they did not present any quantitative models. Beaudeau et al. (2001), in a similar research project on BVD, suggested a linear regression model with high correlation between BTM results and within-herd prevalence of BVD in French dairy herds. However, for BVD, its unstable nature, the variety of clinical manifestations, the abundance of transiently infected cows in infected herds, along with the complications due to active vaccination programs (Brodersen, 2014) would restrict the predictive ability and broad application of the potential models based solely on BTM ELISA values.

Although in several studies, BTM ELISA has been used for classification of herds as non-infected or infected with BLV (Eloit et al., 1990; Sargeant et al., 1997; Sorge et al., 2011; Reber et al., 2012), this was the first time that a quantitative approach for predicting within-herd prevalence of BLV was investigated. Determining the approximate number of infected cows in a herd can be of particular importance when a decision on adopting the most suitable control or eradication strategy is to be made. For instance, if only a few positive cows in a herd were expected, eradication measures such as test and removal could economically be justified. If a low prevalence of the infection (e.g. < 20%) was predicted, then a test and segregation strategy could be desirable (Bartlett et al., 2014). Our models indicated high precision (narrow uncertainty) in the predictions for low-to-medium BTM ELISA titers, giving confidence to farmers who decide to make eradication or segregation decisions. The fluctuations and relatively wider uncertainty for high titers (e.g. PP > 55) should not be concerning to farmers with results

in this range because decision rules (i.e. farm protocols) for farms with estimated prevalence above 30% would be similar; usually including standard management programs in order to reduce the within-herd transmission of BLV (Casal et al., 1990; Bartlett et al., 2014). For these herds with medium-to-high levels of within-herd prevalence, which is the situation for many dairy herds in North America, management strategies without removal or segregation for controlling BLV are preferred due to the considerable cost (and/or impracticality) of removal and segregation schemes (Sandev et al., 2000; Bartlett et al., 2014).

The potential dilution effect from a large number of cows that contribute milk to a bulk tank could theoretically lead to under-estimation of the within-herd prevalence. In our study, however, lactating herd size (representing the potential dilution effect) did not show any significant effect on the within-herd prevalence of BLV. This finding was in agreement with the results from another study by Beaudeau et al. (2001) on BVD. One of the likely reasons for this finding could be relatively small lactating herd size for most of the herds in our study; 78/90 (87%) herds had a lactating herd size of less than 100, and the rest were between 100 to 287. For most Canadian dairy herds, this issue would not be concerning because our study farms were representative of the most dairy farms in Canada with respect to the lactating herd size. The mean lactating herd size in Canada is 70 – 80 cows (Canada dairy-info, 2015), which is very close to the mean of 68 cows in our study herds. However, lactating herd size should be taken into consideration when similar modeling approaches are to be practiced in large dairy herds elsewhere.

5.5.1 Conclusions

Prevalence of infection with BLV at the herd and cow levels on dairy farms of the Maritime region of Canada was very high, indicating the necessity for a broad-based, comprehensive response. Obtaining an estimate of the number of infected cows on a farm would be a fundamental step towards adopting appropriate control or eradication strategies for that farm. The statistical models developed in this study were able to predict true within-herd prevalence of BLV reasonably well, using 2-4 BTM ELISA titers over 2-4 months. However, the choice of one model would primarily depend on different purposes of BTM testing in dairy farms with various levels of BLV infection prevalence (e.g. for reducing high-prevalence of BLV, or eliminating the infection from low-prevalence herds). The presented methodology offers many advantages, including being convenient (BTM samples can easily be accessed), efficient (as few as 2 BTM samples could be used), and inexpensive (individual cow samples not needed). Therefore, the methodology could readily be integrated into future BLV control and surveillance programs. Application of the presented approach could cautiously be extended to dairy farms from other regions with similar structure and management strategies.

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Table 5.1. Herd-level prevalence of infection with bovine leukemia virus for all 623 dairy herds from the Maritime region of Canada, by province (2013).

Province	No. of (tested) herds		No. of positive herds [a]		Herd level prevalence (%)[b]	
	All	DHI [c]	All	DHI	All	DHI
New Brunswick	202	142	179	120	88.6	84.5
Nova Scotia	237	154	219	141	92.4	91.6
Prince Edward Island	184	114	168	102	91.3	89.5
Total	623	410	566	363	90.8	88.5

[a] A herd was positive if any of the 3 monthly BTM ELISA values was equal or larger than 5.
[b] Number of positive herds/number of herds (a census).
[c] Only herds registered on dairy herd improvement (DHI) programs (410/623).

Table 5.2. Concordance correlations among all rounds of bulk-tank milk ELISA results for infection with bovine leukemia virus in Canadian Maritime dairy herds.

CCC (SE)[a]	All 623 herds	90 selected herds [b]
Rounds 1 & 2	0.822 (0.012)	0.856 (0.028)
Rounds 1 & 3	0.723 (0.019)	0.766 (0.044)
Rounds 1 & 4	-	0.732 (0.048)
Rounds 2 & 3	0.805 (0.014)	0.822 (0.034)
Rounds 2 & 4	-	0.751 (0.042)
Rounds 3 & 4	-	0.800 (0.037)

[a] Concordance correlation coefficient and the corresponding standard error.
[b] In the 90 selected herds, there was an additional round of sampling (round 4).

Table 5.3. Final linear regression models for predicting within-herd prevalence of infection with bovine leukemia virus (Y), using different combinations of bulk-tank milk ELISA results (X) in 90 study dairy herds.

Scenario of interest	Final model [a]	SE for slope [b]	R^2 (%)[c]
Only 4 (X_1)	$Y = (0.00773\ X_1 + 0.11121)^2$	0.00065	69
Mean of 4 & 3 (X_2)	$Y = (0.00895\ X_2 + 0.05706)^2$	0.00040	75
Mean of 4 & 3 & 2 (X_3)	$Y = (0.00945\ X_3 + 0.04718)^2$	0.00050	78
Mean of 4 & 3 & 1 (X_4)	$Y = (0.00944\ X_4 + 0.02877)^2$	0.00046	82
Mean of all 4 (X_5)	$Y = (0.00956\ X_5 + 0.03636)^2$	0.00044	82
Mean of 3 & 2 & 1 (X_6)	$Y = (0.00899\ X_6 + 0.08282)^2$	0.00046	76

[a] Y: predicted true within-herd prevalence; and X: BTM ELISA titer (mean of titers from different rounds).
[b] Standard error for the corresponding coefficient of X (slope) in the final models, on square root transformed scale. The coefficients were all highly significant ($P < 0.001$).
[c] Coefficient of determination in percentage.

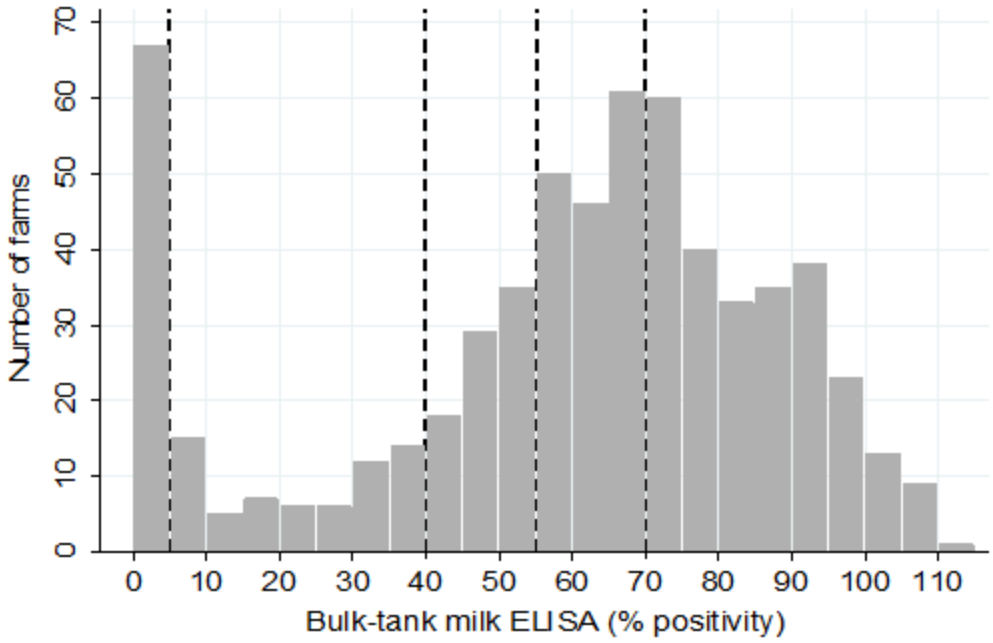

Figure 5.1. Frequency distribution of the mean of three monthly bulk-tank milk ELISA results (percent positivity) for infection with bovine leukemia virus in 623 dairy farms from the Maritime region of Canada (2013). Four dashed lines indicate the cut-points for categorizing the herds into the five prevalence-level groups.

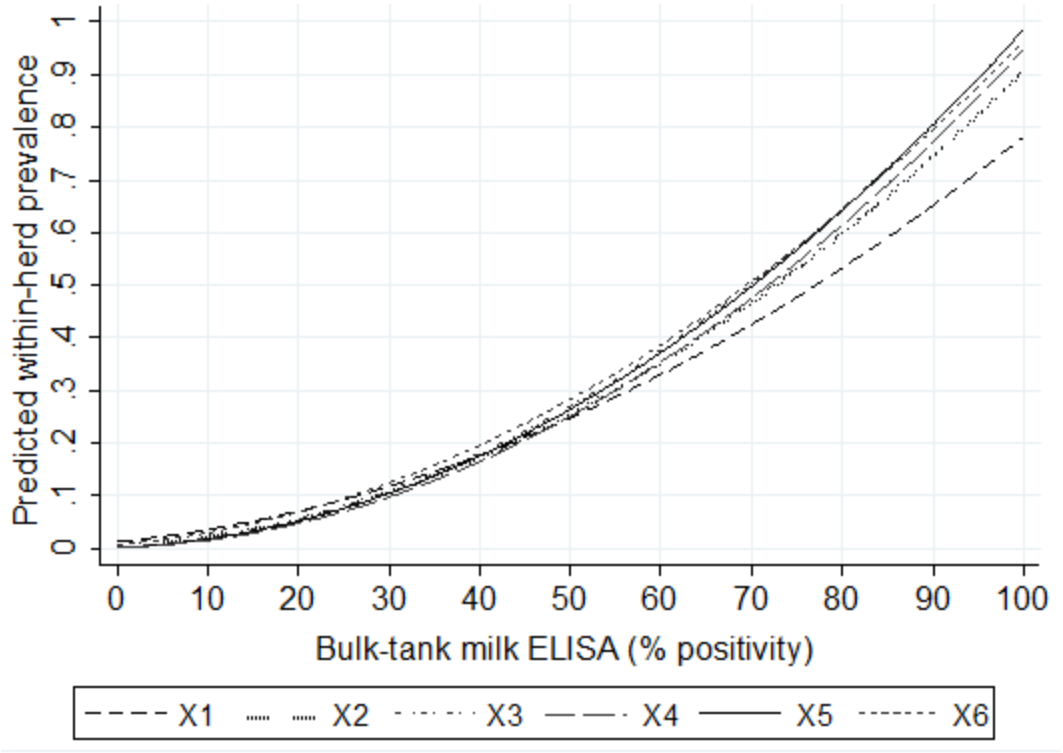

Figure 5.2. Predictive functions for five final models predicting within-herd prevalence of infection with bovine leukemia virus (Y axis), versus six different scenarios of repeated bulk-tank milk ELISA titers (percent positivity; X axis), in 90 selected farms from the Maritime region of Canada. X1: using round 4 scenario; X2: mean of rounds 3 and 4; X3: mean of rounds 2, 3, and 4; X4: mean of rounds 1, 3, and 4; X5: mean of rounds 1, 2, 3, and 4; X6 mean of rounds 3, 2, and 1. Note that X3 and X5 scenarios are not distinguishable (mostly overlapping).

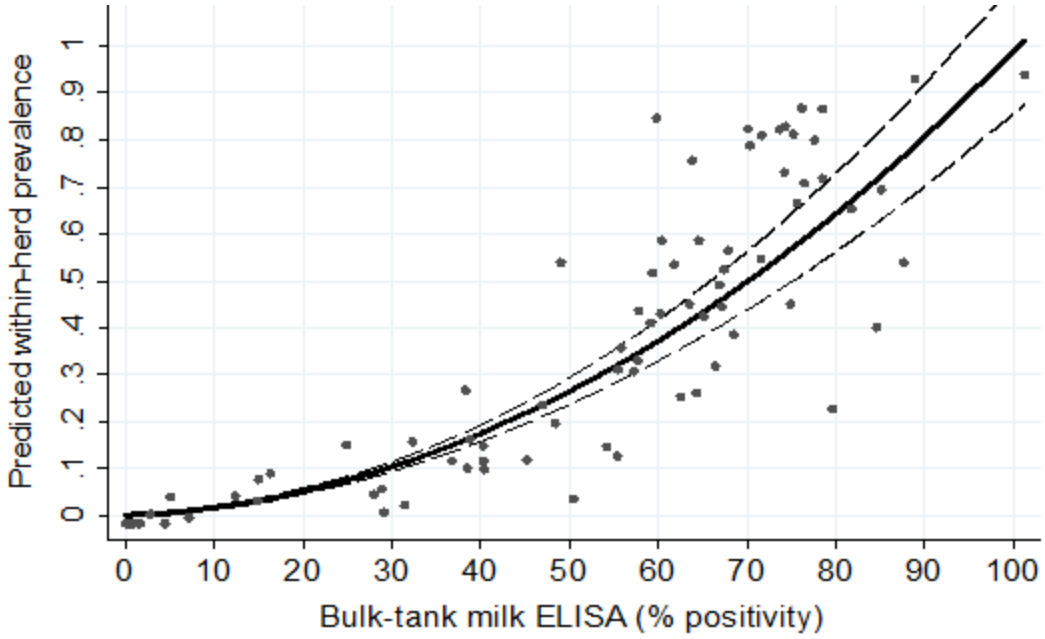

Figure 5.3. Scatter diagram for prediction of the within-herd prevalence of infection with bovine leukemia virus (Y axis), versus the mean value of the four available repeated bulk-tank milk ELISA titers (percent positivity; X axis); from the equation: Y = (0.00956 X5 + 0.03636)2 (scenario X5). Dashed lines around the solid prediction-line represent the 95% confidence interval for the prediction (after back-transformation to original scale). Each point represents one of the 90 selected farms from the Maritime region of Canada.

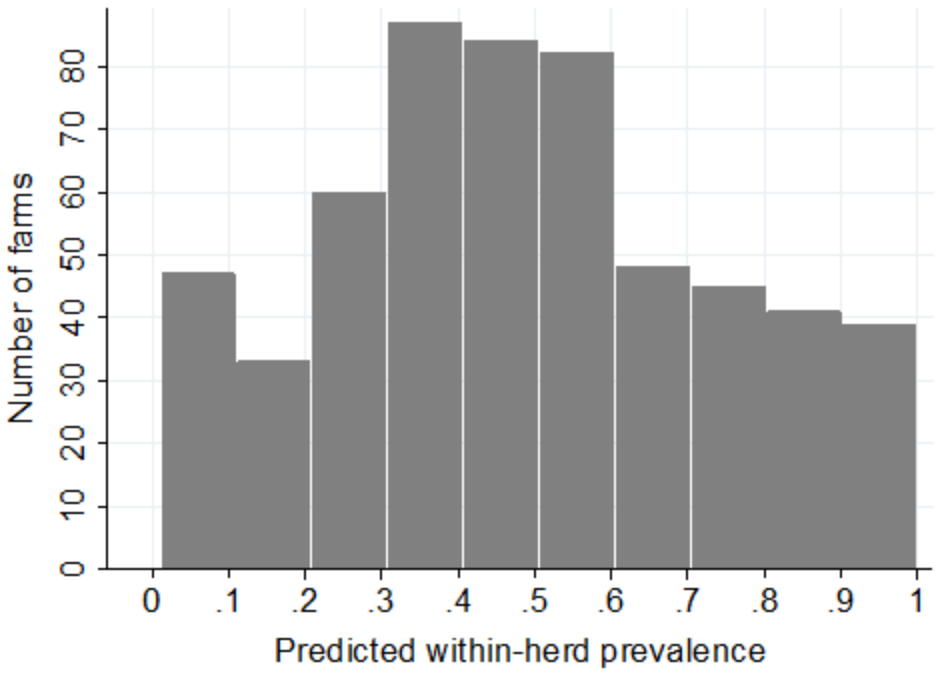

Figure 5.4. Frequency distribution of the predicted within-herd prevalence of infection with bovine leukemia virus (based on the corresponding model, X_6) for 566 infected dairy farms from the Maritime region of Canada.

CHAPTER 6

DIAGNOSTIC PERFORMANCE OF AN INDIRECT ELISA TO DETECT BOVINE LEUKEMIA VIRUS ANTIBODIES IN BULK-TANK MILK SAMPLES

This chapter has been accepted for publication in the Canadian Veterinary Journal as a brief communication (without substantive change):

"Nekouei, O., J. Durocher and G. Keefe. 2015. Diagnostic performance of an indirect ELISA to detect bovine leukemia virus antibodies in bulk-tank milk samples. Can. Vet. J."

6.1 Abstract

The objective of this study was to assess the diagnostic performance of a commercially available ELISA for detecting bovine leukemia virus (BLV) antibodies in bulk-tank milk (BTM) samples from Eastern Canada. The study population consisted of 133 dairy farms, including 8358 lactating cows, from provinces of Prince Edward Island (PE), New Brunswick (NB), Nova Scotia (NS), and Quebec (QC). Within every herd, one BTM sample and samples from all cows that contributed milk to the BTM on that day were collected and tested for BLV-antibodies by an ELISA. One hundred and eight tested herds (81.2%) were found to be truly infected, based on the individual cow test results. Using estimated true within-herd prevalences of BLV as the reference standard, sensitivity and specificity of the BTM test at the determined optimum cut-point of 5 (percent positivity) were estimated at 97.2% (92.1 – 99.4%) and 100% (86.3 – 100%), respectively. The test was therefore recommended as a valid, cost-effective tool for large-scale BLV screening and monitoring schemes.

6.2 Introduction

Enzootic bovine leukosis (EBL) is an economically important disease of dairy cattle caused by bovine leukemia virus (BLV). The virus is transmitted through infected blood lymphocytes. Premature culling, death, and condemnation of carcasses at slaughter due to lymphoma, abnormal immune function, as well as restrictions on international trade of infected cattle and their products are among the most significant economic losses attributed to the disease (Sandev et al., 2000; Bartlett et al., 2014).

Many European countries are now officially free from EBL, whereas prevalence of the infection in North America has been high and appears to have a rising trend

(Bartlett et al., 2014; Chapter 5). For instance, in the Maritime region of Canada, herd-level prevalence of the infection from 70% in 1998 (VanLeeuwen et al., 2001) reached to over 90% in 2013 (Chapter 5).

Using bulk-tank milk (BTM) samples has become one of the most convenient and economically efficient procedures for screening for important diseases in dairy herds, including EBL (Sargeant et al., 1997b; Sorge et al., 2011b). Among available commercial tests for detection of BLV-antibodies, milk ELISA has been documented as a desirable method with great performance in large-scale surveillance programs (Gutierrez et al., 2001; Erskine et al., 2012c). However, applying commercial ELISA tests, particularly to the pooled samples (e.g. BTM), could lead to variable levels of uncertainty in the results. Several factors, including study region, herd (pool) size, sampling procedures, and transfer process can potentially contribute to the variable test results. Therefore, it has been recommended that the validity of diagnostic tests should be evaluated in different populations before integrating the tests in large-scale control and eradication programs (Christensen and Gardner, 2000; Greiner and Gardner, 2000). The objective of this study was to assess the diagnostic performance (sensitivity and specificity) of a commercially available ELISA for detecting BLV-antibodies in BTM samples from Eastern Canada; in order to validate the routine application of this test to the BLV monitoring programs implemented in the region.

6.3 Materials and methods

6.3.1 Study population and sample collection

The study population consisted of 133 dairy herd improvement (DHI)-registered dairy herds, including 8358 lactating cows, from four eastern provinces of Canada

(Prince Edward Island (PE), New Brunswick (NB), Nova Scotia (NS), and Quebec (QC)). During 2013, 30 farms were randomly selected from each of PE, NB, and NS (a total of 90 farms) based on a wide range of BLV within-herd prevalences (Chapter 5). In 2014, a similar study was carried out on 43 purposively selected dairy farms from Quebec. The Quebec herds were also selected from a potential wide spectrum of prevalences, according to the available data from historic surveys. One BTM sample was obtained from every selected farm, and on the same day of the BTM sampling, all lactating cows that contributed milk to the BTM were also individually sampled via the corresponding milk meters.

6.3.2 Laboratory testing

Individual cow and BTM samples (30 ml each) from PE, NB, and NS were transferred to the Maritime Quality Milk (MQM) laboratory located in the University of Prince Edward Island, in Charlottetown to be tested for BLV-antibodies. All Quebec samples were submitted to the Valacta central laboratory (Sainte-Anne-de-Bellevue, QC) for BLV testing. All samples were preserved with BROTAB (Sierra Court, CA, USA) and tested in a maximum of seven days from the original sampling dates using a commercial indirect ELISA (Svanovir BLV gp51-Ab, Svanova, Uppsala, Sweden). The test results were reported as percent positivity (PP) values [PP = ($OD_{corrected}$ sample/$OD_{corrected}$ positive control) × 100, where OD = optical density].

6.3.3 Statistical analyses

All of the statistical analyses were conducted in Stata 13.1 (StataCorp, College Station, TX. USA).

To determine the apparent within-herd prevalence of BLV-antibodies (AP), number of positive cows (PP > 45) was divided by the number of lactating (tested) cows for every herd. True within-herd prevalence of BLV infection (TP) was then estimated for each of the selected herds using the following formula (Dohoo et al., 2009):

$TP = (AP + Sp - 1)/(Se + Sp - 1)$

Where AP is the apparent prevalence of BLV infection within the study herds; sensitivity (Se) and specificity (Sp) of the individual ELISA test at the applied threshold of 45 were 95.5% and 98.4%, respectively (Chapter 4). The true within-herd prevalence was regarded as the reference standard for evaluating the diagnostic performance of the pooled-level application of the BTM ELISA. If the true within herd prevalence was zero (i.e. all cows were negative), the herd was considered as uninfected; and if it was above zero (i.e. at least one positive cow was present), the herd was considered as infected with BLV. A two-graph receiver operating characteristic (ROC) analysis was carried out to determine the optimal cut-point on BTM ELISA values using the defined dichotomized reference standard.

6.4 Results

Based on the true within-herd prevalence of BLV, 81.2% (108/133) of the study herds were found to be infected (i.e., true positive herds). Of 8358 tested cows (90% Holstein), 2661 (31.8%) were positive to BLV milk-antibodies in the individual cow testing. Descriptive statistics for the study herds, by province, are presented in Table 6.1. The mean of BLV true within-herd prevalence for the 108 infected herds was 0.39 (SD = 0.27). Figure 6.1 displays the distribution of BLV true within-herd prevalence for the study herds.

From the two-graph ROC analysis (Figure 6.2), maximum accuracy for BTM ELISA titers was achieved at 2.1 and 7.2 (percent positivity), respectively. Hence, the midpoint of 5 (-also recommended by the manufacturer of the test kit) was considered as our practical cut-point value. At this cut-point, sensitivity and specificity of the BTM ELISA were estimated at 97.2% (95% CI: 92.1 – 99.4%) and 100% (95% CI: 86.3 – 100%), respectively (Table 6.2).

6.5 Discussion

According to our established reference standard, three truly infected herds were tested negative by the BTM ELISA (defined as false negative herds). Each of those 3 herds harbored only one infected cow: one herd from NS (including 53 lactating cows), and two herds from QC (including 37 and 47 lactating cows). In stringent eradication programs, repeated sampling from bulk-tank over appropriate time intervals has been recommended in order to compensate for the imperfect sensitivity of the BTM tests, and to capture as many positive animals in a herd as possible (Dohoo et al., 2009).

Addition or elimination of some cows can be influential on the BTM titers, such as those at advanced stages of BLV infection (e.g. cows with persistent lymphocytosis), because they often produce high levels of virus and circulating antibodies (Juliarena et al., 2007). However, it is generally believed that the prevalence of BLV in herds from endemic areas (e.g. North America) remain fairly steady over time (Radostits et al., 2006; Chapter 5); this characteristic supports the validity of the current testing strategies (BTM ELISA) used in detecting BLV infection.

We were not able to apply more sophisticated statistical analyses to our data in order to include some potentially important herd-level factors such as lactating herd size

(surrogating the potential dilution effect of BTM) because there were only 3 false diagnoses by the BTM test. However, this issue should not lead to any substantial bias since the selected herds were reasonably representative of the herds in Eastern Canada with regards to the main characteristics, including herd size. For instance, the average lactating herd size in our study was 62.8, which was very close to the average of lactating herd size in all four study provinces (approximately 60).

6.5.1 Conclusions

Applying a cut-point of 5 to the ELISA test, when it was used on BTM samples generated reasonably valid results. Producers whose farms are free from BLV and would desire to maintain their negative status or those who wish to monitor their decreasing trend of BLV prevalence on their farms (due to taking control measures) could efficiently adopt ongoing monitoring using the BTM ELISA test over appropriate time-intervals. Application of the BTM ELISA in other regions (particularly with large herds) should be validated before applying to future surveillance programs.

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Table 6.1. Descriptive summary for 133 study dairy herds that had all their lactating cows tested for bovine leukemia virus milk-antibodies, from eastern provinces of Canada.

Province [a]	Tested herds	Lactating herd size			Tested cows	Positive cows [a]	Proportion (%) [b]
		Min	Median	Max			
New Brunswick	30	23	51	287	2232	845	37.8
Nova Scotia	30	30	65.5	214	2281	460	20.2
Prince Edward Island	30	28	52	126	1598	555	34.7
Quebec	43	14	56	145	2247	801	35.6
Total	133	14	52	287	8358	2661	31.8

[a] Positive in individual milk ELISA test (percent positivity > 45).
[b] Number of positive cows/Number of tested cows.

Table 6.2. Cross-classification of bulk-tank milk ELISA results at the cut-point of 5 PP (percent positivity) for antibodies against bovine leukemia virus (BLV) and true within-herd prevalence of BLV in 133 study herds[a].

	True within-herd prevalence (reference)		Total
BTM ELISA	> 0	= 0	
PP ≥ 5	105	0	105
PP < 5	3	25	28
Total	108	25	133

[a] Sensitivity and specificity of the BTM ELISA were estimated at 97.2% (92.1 – 99.4%) and 100% (86.3 – 100%), respectively.

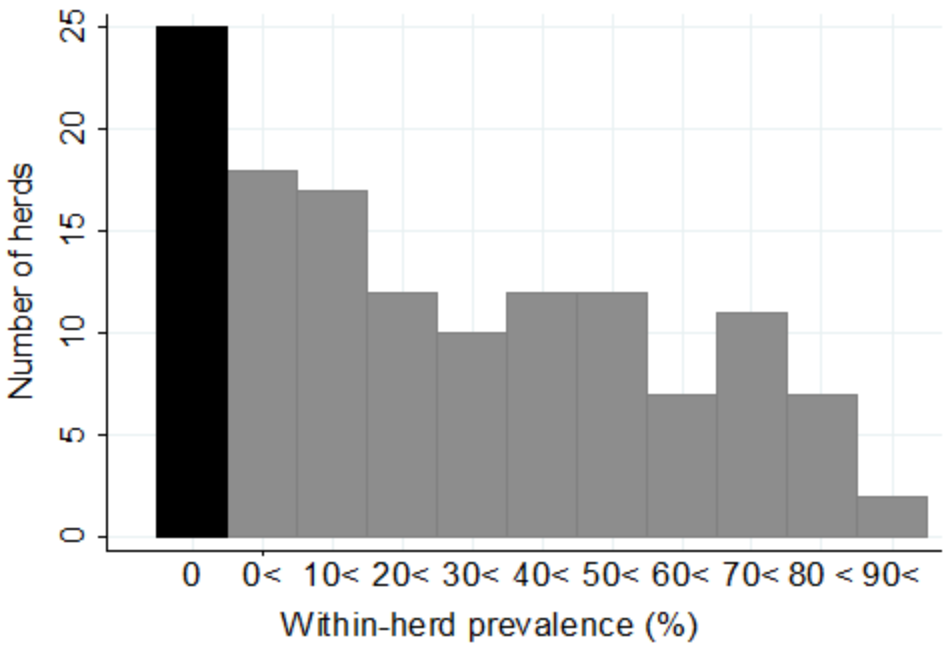

Figure 6.1. Frequency distribution of the within-herd prevalence of infection with bovine leukemia virus for 133 study herds from eastern provinces of Canada. The black bar represents uninfected herds (25/133).

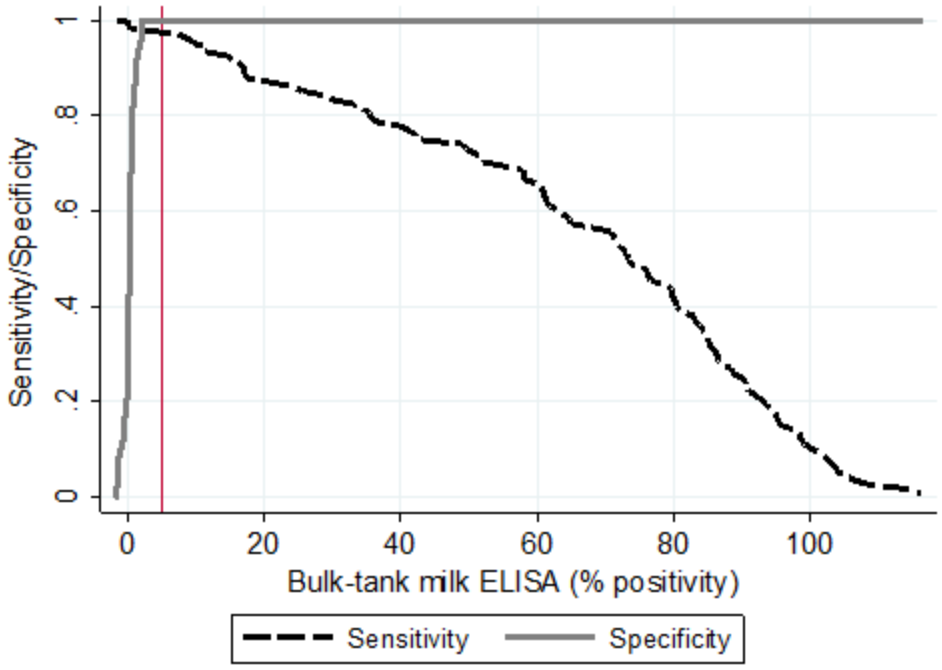

Figure 6.2. Two-graph receiver operating characteristic curve (ROC) illustrating sensitivity and specificity versus all possible cut-off values for bulk-tank milk ELISA results (percent positivity) for bovine leukemia virus antibodies in 133 study herds. True within-herd prevalence was used as the dichotomized reference standard (0; or 1 if it was> 0). The vertical solid line represents the recommended cut-off of 5.

CHAPTER 7

OVERALL CONCLUSIONS

Enzootic bovine leukosis (EBL) is an important infection of dairy cattle which is caused by bovine leukemia virus (BLV) (Radostits et al., 2006; Smith, 2009). Infection with BLV imposes substantial financial loss to the dairy industry, particularly in countries with high prevalence of the infection (e.g. Canada). Major monetized losses from the infection include premature culling, death, and condemnation of carcasses at slaughter due to lymphoma, production loss, lower reproductive efficiency, impaired immune function and susceptibility to other infections, as well as trade restrictions imposed on infected cattle and their products (Sandev et al., 2000; Bartlett et al., 2014).

Prevalence of the infection in Canadian dairy herds and cows is high (over 80% and 30%, respectively) and continues to increase (Sargeant et al., 1997; VanLeeuwen et al., 2001; VanLeeuwen et al., 2005; VanLeeuwen et al., 2006; Scott et al., 2006; Chapter 5). Nevertheless, there are no broad-based, national programs in place for controlling EBL in Canada.

Overall, the objectives of the research described in this thesis were to: 1) identify important herd-level risk factors for BLV infection in Canada (Chapter 2); 2) assess lifetime effects of the infection on milk production and longevity of Canadian dairy cows (Chapter 3); 3) evaluate the potential carryover of BLV antibodies in shared milk meters

(Chapter 4); 4) to address the carryover issue by adjusting assay cut-off values in screening programs (Chapter 4); 5) to determine the current herd-level prevalence of the infection in the Maritime region (Chapter 5); 6) to develop applied statistical models for predicting within-herd prevalence of BLV using bulk-tank milk (BTM) antibody levels (Chapter 5); 7) to validate diagnostic performance of a BTM ELISA in dairy herds from eastern Canada (Chapter 6).

All of the findings from the described work could eventually contribute to designing and conducting efficient control/management programs for BLV infection in Canada.

7.1 Herd-level risk factors for BLV infection

The focus for Chapter 2 was to identify potentially important risk factors for BLV infection in Canadian dairy herds, as a prerequisite to developing an effective control program. Of 272 herds which successfully completed the study, 78% were identified as BLV-positive. Among more than 15 evaluated determinants for the infection, a few of those were significantly associated with BLV seroprevalence at the herd-level. Herds with clinical cases of leukosis during the 12 months prior to sampling, as well as herds which purchased animals with unknown BLV infection status in the last five years, had a significantly greater proportion of BLV-positive animals. In the western provinces of Canada, changing gloves between cows during pregnancy examination was not statistically associated with lower seroprevalence of BLV compared with not changing gloves. In another part of the analyses, herds from eastern Canadian provinces and those not purchasing cows in the last five years were more likely to be free from BLV.

In general, moving towards closed herds in the cattle industry will be an efficient way to control different contagious diseases (including EBL), wherever possible. National, regional, and herd-specific BLV control programs should not only be focused on inhibiting virus transmission between herds by purchasing BLV-negative replacement animals, but also focused on decreasing BLV spread between cows (i.e. within a herd), particularly in herds with high prevalence of BLV infection. Applying proper count data models is recommended to extract all of the available information in different data sets on risk factors for infectious diseases of dairy cattle.

7.2 Lifetime effects of BLV infection on longevity and milk production

The objective in Chapter 3 was to determine the effects of BLV infection on lifetime milk production and longevity of dairy cows in Canada. All participant cows had been culled or died by the onset of the conducted historical cohort study. Overall, 4052 cows from 348 herds were enrolled in the study. In the longevity part of this Chapter, the interaction term between time (the number of life lactations) and BLV-status was significant. Positive cows to BLV had constantly greater probability of being culled (or dying) than the negative cows. In the milk production portion of the study, the interaction term between BLV-status and lifetime lactations of the cows was highly significant, indicating that lifetime BLV effects on the total milk production was dependent on the lactation in which the study cows were culled or died. Only BLV-positive cows with short longevity (2 and 3 lactations) had substantially lower total milk productions compared with their negative counterparts. As the cows lived longer (> 3 life lactations), the differences in lifetime milk production between the two cohorts were no longer substantial.

Regarding the chronic nature and gradual progress of BLV infection, evaluating its economic impacts over the lifetime of dairy cows is very informative. The design of our study was well-suited for describing the potential causal association between BLV infection and the corresponding production and longevity effects. Seropositive cows had consistently shorter lifespan compared with their negative counterparts, suggesting that the infection could be one of the main causes of premature culling (or death) at any age groups of cows.

7.3 Carryover of BLV-antibodies in shared milk meters

The focus for Chapter 4 was two-fold. The first objective was to assess the potential for carryover of antibodies against BLV in milk samples obtained from shared meters. The study included 236 paired milk samples from 8 dairy farms in Prince Edward Island. Two simultaneous milk samples, one hand-collected at the beginning of milking, and the other from the corresponding milk meter were taken from all lactating cows that were milked at the selected meters. The sequence of cows using each meter was recorded. All samples were tested for BLV antibodies using a commercial indirect ELISA. Antibody carryover potential was assessed in meter-collected samples which were preceded by other cows using the same meters. At the standard cut-off value of the diagnostic test (milk ELISA), 46.6% of the hand-collected, and 57.6% of the meter-collected samples were positive. For low-titer cows (e.g. true negatives), the likelihood of antibody carryover significantly increased as the titer of preceding cows increased, while this change was not substantial for high-titer cows. In addition, the odds of obtaining false diagnoses in meter-positive samples became larger with increasing titers in the preceding cows.

With respect to the results from the first part of this Chapter, the second objective was to determine if adjustment of the diagnostic test cut-off value would improve the test characteristics for meter-collected results. For this purpose, a receiver operating characteristic (ROC) curve analysis was carried out to define the optimal cut-point which would result in lower number of false diagnoses. Based on the original kit and the adjusted cut-off values for the meter-collected samples, three categories of ELISA results (negative, suspicious, and positive) were defined and a retest was recommended on the suspicious samples.

Carryover of BLV antibodies at shared milk meters was significant. For low-titer cows (e.g., true negatives), the carryover effect was positively associated with the titer of the preceding cows. This could result in generating false-positive results in the BLV antibody-ELISA test on meter-collected samples from dairy herd improvement (DHI) procedures. For meter-collected samples, if we only rely on the dichotomous test results with the original cut-off, the consequences can be economically substantial. Thus, defining a suspicious category for the ELISA titers and recommending a retest on the samples falling within this range would be very helpful in reducing the false positive rate.

7.4 Predicting within-herd prevalence of BLV infection

The objectives for Chapter 5 were to: 1) determine the prevalence of BLV infection at the herd level using a BTM antibody ELISA in the Maritime region of Canada; and 2) develop applied statistical models for predicting within-herd prevalence of BLV infection using the BTM antibody levels.

To detect BLV infection and the antibody levels, a census was implemented on BTM samples from all dairy farms in the Maritime region (3 rounds of sampling on 623

farms). Another round of BTM sampling was coincided with individual cow sampling (all cows that contributed milk to the fourth BTM) in 90 selected herds. Herd-level prevalence of BLV in the Maritime region was 90.8%. In the individual testing, 30.4% of cows were positive. Prevalence of infection with BLV at the herd and cow levels on dairy farms of the Maritime region of Canada was very high, indicating the necessity for a comprehensive response. Obtaining an estimate of the number of infected cows on a farm would be a fundamental step towards adopting appropriate control or eradication strategies for that farm.

The statistical models developed in this study were able to predict true within-herd prevalence of BLV reasonably well. Predictions from the models were particularly accurate for low-to-medium spectrums of the BTM titers. The model including all BTM tests (4 rounds of sampling) as the predictor had the best fit, although the models using 2 and 3 BTM tests provided similar results to 4 repeated tests. However, the choice of one model would primarily depend on different purposes of BTM testing in dairy farms with various levels of BLV infection prevalence (e.g. for reducing high-prevalence of BLV, or eliminating the infection from low-prevalence herds). The presented methodology offers many advantages, including being convenient (BTM samples can easily be accessed), efficient (as few as 2 BTM samples could be used), and inexpensive (individual cow samples not needed). Therefore, in order to motivate dairy producers to take decisive control measures against BLV, the methodology could readily be integrated into future BLV control and surveillance programs. Application of the presented approach could cautiously be extended to dairy farms from other regions with similar structure and management strategies.

7.5 Diagnostic performance of an ELISA for herd-level application

The focus for Chapter 6 was to assess the diagnostic performance (sensitivity and specificity) of a commercially available ELISA for detecting bovine leukemia virus (BLV) antibodies in bulk-tank milk (BTM) samples collected from dairy herds in eastern Canada. Of 133 tested herds from eastern Canada, 108 herds were found to be truly infected. Of 8358 tested lactating cows, 31.8% were positive to BLV milk antibodies in the individual cow testing. The optimal cut-point for BTM ELISA titers was achieved at 5 percent positivity. At this cut-point, sensitivity and specificity of the BTM ELISA were estimated at 0.972 (95% CI: 0.921 – 0.994) and 1 (95% CI: 0.863 – 1), respectively.

Application of the ELISA test to BTM samples at the cut-point of 5 (which was also the manufacturer's recommendation) generated reasonably valid results. Farmers whose farms are free from BLV and would desire to maintain their negative status or those who wish to monitor their decreasing trend of BLV prevalence on their farms (due to taken control measures) could efficiently adopt ongoing monitoring using the BTM ELISA test over appropriate time-intervals. Application of the BTM ELISA in other regions (with large herds in particular) should be validated before applying to future surveillance programs.

7.6 A provisional example for a comprehensive control/eradication program

Combining all of the findings from this thesis with the results from historic studies, we could design and conduct a comprehensive control/eradication program for BLV at herd, regional, or even national level in Canada. A provisional example for such a program is demonstrated in Figure 7.1. From the figure, the following steps could be taken towards effective control and finally eradication of BLV:

1. Preliminary screenings for antibodies against BLV using BTM ELISA (Svanova test kit, Chapters 4-6); BTM sampling and testing are repeated on a regular basis (recommendation: at least 2 or 3 samples with 2-month intervals);

2. If the results (from Step 1) are negative, BTM ELISA monitoring is continued on a regular basis with appropriate time intervals. Meanwhile, rigorous biosecurity measures must be taken to inhibit the introduction of BLV to these negative herds. The consistently negative herds may pursue a BLV-free certificate;

3. If the results (from Step 1) are positive, the developed statistical models in Chapter 5 will be applied to estimate the within-herd prevalence of the infection;

4. If the within-herd prevalence is at a very low level (only a few positive animals are present in the herd), test and removal strategy may be justified (Section 1.1.7). For this purpose, individual animal sampling and testing (milk and serum ELISA) would be necessary. The infection could be eliminated from these herds and then they could join the negative category of herds;

5. If the within-herd prevalence is at relatively low levels (e.g. < 20%), test and segregation strategy could be adopted. For this purpose, individual animal sampling and testing (milk and serum ELISA) would be necessary. Segregation of positive cows from the negative ones is not always a practical option in some herds in Canada. In this case, one recommendation could be finding and separating cows, which potentially pose a greater risk to their susceptible herd mates (e.g. PL cows). To detect these animals, repeated testing, along with differential blood cell count on cows with high levels of antibodies could be very helpful. Segregating cows with consistently high levels of

antibodies and/or PL cows is recommended (when this strategy is not justifiable to be extended to all of the infected cows within a herd);

6. If the within-herd prevalence is estimated at medium levels or higher (e.g. > 20), suitable management interventions which can inhibit the potential movement of contaminated blood between infected and non-infected animals (such as using single-use needles and rectal sleeves, as well as purchasing BLV-negative cows; Chapters 1 and 2) are recommended. In this case, the goal would be to decrease the within-herd prevalence of BLV during a reasonable time period (e.g. 2 years). In order to assess the efficacy of the adopted measures, regular BTM screening (as explained above) would be necessary. Subsequent to reducing the prevalence to low levels, the approach explained in Step 5 could be pursued;

7. Evaluations of the efficacy and the corresponding cost-benefit analyses would be imperative at different steps of this comprehensive control/eradication program.

7.7 Current research limitations and future directions

Overall, with respect to the high prevalence of BLV infection across Canada, the outlined adverse economic impacts, and its increasing importance, pursuing broad-based, efficient control programs is necessary. The final goal of this thesis was to lay a proper foundation for developing efficient control and eradication programs for BLV in Canada. Despite the accomplishments of the current work, there are still some challenges and uncertainties regarding BLV infection control.

Herd-level risk factors and economic impacts of BLV infection (presented in Chapters 2 and 3) were based upon the diagnostic tests, which were carried out between 1998 and 2003. There is always a need for real-time evaluations of the economic impacts

of the infection in a specific herd or region given the fact that most control and eradication options are highly dependent on the within-herd prevalence of the infection, value of cows within a herd, and the management structure of the herd (Radostits et al., 2006; Bartlett et al., 2014). In order to make optimal decisions on adopting the most suitable strategy against BLV, estimating the within-herd prevalence and implementing a cost-benefit analysis in the real-time (i.e. before and concurrent with an undertaken control/eradication program) are imperative.

Growing concerns regarding carryover or cross-contamination of the samples from shared milking equipment was addressed in Chapter 4. Over the last several years, meter-collected samples have been used to reduce the cost and time in screening for important diseases of dairy cattle, and this utilization is increasing. Our findings on carryover of BLV-antibodies could logically be extended to other commonly tested pathogens. Therefore, further similar studies focusing on each specific diagnostic target (e.g. antibodies against a particular pathogen) are essential to the success of related surveillance programs. The carryover effects should be measured and addressed in a distinct way for each pathogen, based on its characteristics, the diagnostic tests used, and the corresponding program. Adjusting the assay cut-off values could be one of the most practical approaches for reducing the false diagnostic rates due to carryover.

The prediction models developed in Chapter 5 could readily be integrated in the future surveillance programs for BLV. Hence, it could act as compelling motivation for producers to take some rigorous measures while saving a great deal of time, effort, and cost. The statistical models were well-suited for the herds from Atlantic Canada. To generate the most efficient models, with respect to the dairy herd structure and

management practices in other regions (or countries), similar research on developing and validating such predictive models is recommended.

Appropriate diagnostic tests play a critical role towards ensuring the application and success of any surveillance programs. For BLV, milk-ELISA is the commonly used test with excellent accuracy at individual level application. Our findings in Chapter 6 emphasized the importance of validating the screening tests. When a diagnostic setting (e.g. a commercial test kit) is to be applied to a particular region (or country) on large-scale for the first time, assessing its validity on a representative sample of herds could be very informative and substantially reduce the costs associated with potential false diagnoses.

Using the obtained results from this thesis and preceding studies (VanLeeuwen et al., 2001; VanLeeuwen et al., 2005; Tiwari et al., 2005; Scott et al., 2006; Tiwari et al., 2007; Vanleeuwen et al., 2010; Sorge et al., 2011), a reasonably strong foundation has been laid towards defining and conducting cost-efficient control programs for BLV in Canada. Subsequent to the implementation of future programs, additional longitudinal studies on their efficacy will be necessary. For instance, if a producer (who has a high-prevalence dairy herd) is implementing some recommended control measures (e.g. purchasing negative animals, using single-use needles and obstetric sleeves, etc.) to reduce the prevalence of BLV infection on his farm, he could monitor the antibody levels of BTM on a regular basis (according to our developed methodology/models in Chapter 5). With this, he would be able to assess the efficacy of the adopted strategies in decreasing the level of prevalence over defined time intervals (e.g. during 2 years or more).

Producing efficient vaccines against BLV could be a real advancement in the future control campaigns. Many previous attempts to develop an effective vaccine against BLV have been unsuccessful, mostly due to incomplete or transient stimulation of the host immune response. Some promising results have recently been achieved in developing attenuated clone (but replication competent) that protects against the virus in dairy herds (Gutiérrez et al., 2014; Frie and Coussens, 2015). An ideal BLV vaccine would have to be non-infectious, non-oncogenic, and should not interfere with the tests commonly used to detect the infection (Radostits et al., 2006). Further studies on different aspects of the vaccine development would be immensely beneficial to pursuing efficient control and eradication programs in Canada and other endemic areas.

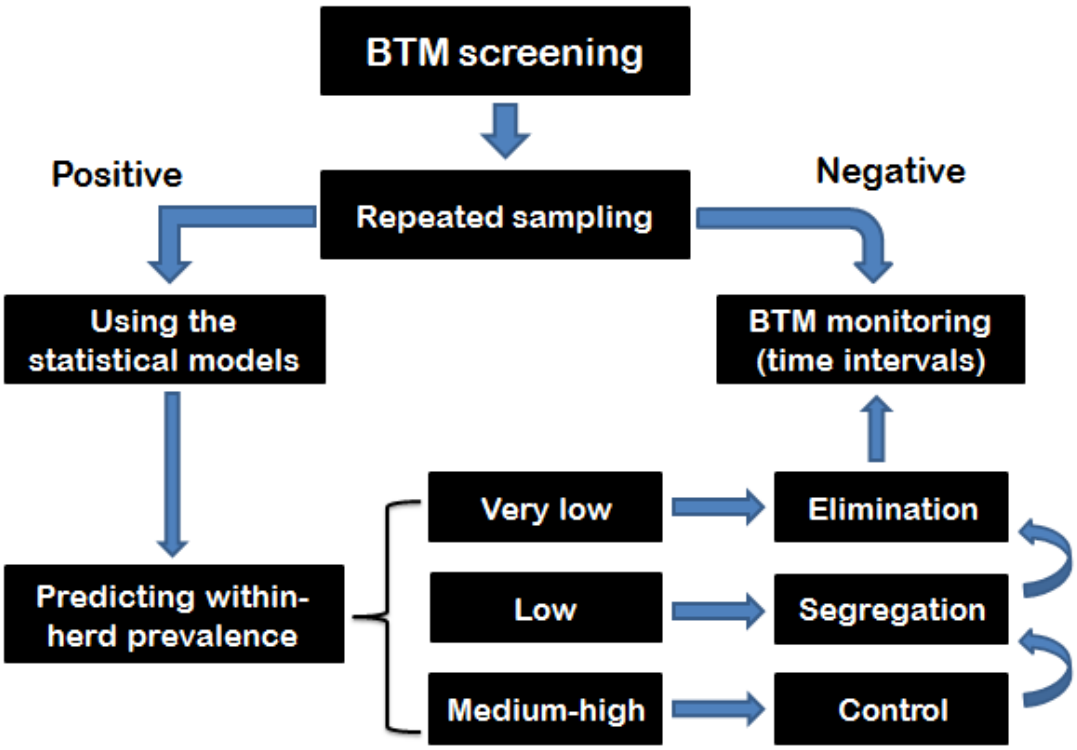

Figure 7.1. A provisional example of a comprehensive control/eradication program for enzootic bovine leukosis designed using the findings from the current research and historic studies in Canada. This program is based on bulk-tank milk (BTM) screening and monitoring for bovine leukemia virus antibodies applying the test kit and methodology outlined in Chapter 5. Please visit Section 7.7 for the step-by-step explanations.

7.7 References

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APPENDIX A

The political map of Canada, displaying all provinces (10), and territories (3). Adapted from http://en.wikipedia.org/wiki/File:Political_map_of_Canada.png (last access on September 19, 2015).

APPENDIX B

Prediction of the within-herd prevalence of infection with bovine leukemia virus (Y axis), versus the repeated bulk-tank milk ELISA titers (percent positivity; X axis), from four developed models in the study (scenarios X_1, X_2, X_4, and X_6). Dashed lines around the solid prediction-lines represent the 95% confidence intervals for the predictions (after back-transformation to original scale). Each point represents one of the 90 selected farms from the Maritime region of Canada. X1: using concurrent scenario; X2: mean of rounds 3 and 4; X4: mean of rounds 1, 3, and 4; X6: mean of rounds 1, 2, and 3.